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**CHARACTERIZATION AND
MODULATION OF HISTAMINERGIC
AND NON-HISTAMINERGIC ITCH**

**BY
GIULIA ERICA ALIOTTA**

DISSERTATION SUBMITTED 2022



AALBORG UNIVERSITY
DENMARK

CHARACTERIZATION AND MODULATION OF HISTAMINERGIC AND NON-HISTAMINERGIC ITCH

by

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AALBORG UNIVERSITY
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Dissertation submitted

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CV

Giulia received a B.Sc. degree in biology, and a M.Sc. in biology applied to biomedical research from University of Milan, Milan, Italy. Hereafter she enrolled as a PhD fellow under the supervision of Prof. Lars Arendt-Nielsen at Center for Neuroplasticity and Pain, Aalborg University, Denmark.

During the three years of PhD, her main interest has been the study of human somatosensation, with special focus on itch and related dysesthesias. Her current research involves the characterization and modulation of histaminergic and non-histaminergic itch, by using human surrogate models, quantitative sensory tests and vasomotor imaging. She presented her results on several international conferences as abstracts and poster presentations and she published them in international peer-reviewed journals. She has also been involved in supervision of different groups of students in 7th, 8th, and 10th semester.

ENGLISH SUMMARY

Chronic itch is a symptom of many dermatological diseases (atopic dermatitis, psoriasis, urticaria), but it can also be present in non-cutaneous conditions (cholestasis, renal insufficiency, peripheral neuropathy). Itch severely affects the quality of life of the patients. In fact, it interferes with important functions such as concentration, sleep quality, sexual activity, etc., and is known to increase depression and anxiety symptoms. Until now, the lack of knowledge on the possible itch and pain interaction precludes an efficient and curative therapy.

Itch and pain share many similarities in terms of neuronal mediators, receptors, and patterns of neuronal sensitization, but the overlap between these two sensations needs to be better understood. The cutaneous pruriceptive subpopulations that transmit itch are part of a larger population of fibers that also respond to noxious stimuli. Itch can be divided into two distinct pathways depending on which subpopulation is activated, defined as histaminergic and non-histaminergic itch pathways. Non-histaminergic itch is particularly important because it is the most difficult to treat, by also being refractory to antihistamines. In human surrogate models of itch, two molecules are used to activate the two pathways: histamine and cowhage. These models are useful to explore characteristics of itch and search for new targets for therapy, even though they present some limitations. Another model of non-histaminergic itch involves the application of bovine adrenal medulla (BAM)8-22. BAM8-22 and cowhage induce histamine-independent itch by the binding of two different families of receptors present on the surface of the same fibers.

The actual treatments for itch are several and they target different aspects of itch transmission. One of these is doxepin cream, a tricyclic antidepressant with strong anti-histaminergic properties. Another is EMLA cream, a local anesthetic that blocks the sodium channels.

Considering that, the aims of this PhD-project were to:

- Assess the properties of a model of non-histaminergic itch based on bovine adrenal medulla (BAM) 8-22;

- Compare the effect of doxepin cream on histaminergic and non-histaminergic itch induced by histamine, cowhage, and BAM8-22;
- Investigate the effect of repetitive application of EMLA cream on neurogenic inflammation, mechanical and thermal sensitivities, and histaminergic and non-histaminergic itch induced by histamine and cowhage.

The first study confirmed that BAM8-22 is a good model of non-histaminergic itch without inducing any pain. This is relevant because BAM8-22 and cowhage therefore show different properties, where cowhage induces pain. The second study compared itch induced by histamine, BAM8-22, and cowhage alone and with pretreatment with doxepin cream. Interestingly, doxepin not only almost abolished histaminergic itch, but also reduced the non-histaminergic. In the last study, repetitive application of EMLA cream was conducted and different aspects were evaluated. EMLA cream reduced mechanical and thermal sensitivities without a dose-cumulative effect. Moreover, EMLA reduced non-histaminergic itch without affecting the histaminergic pathway.

In conclusion, BAM8-22 is a valid surrogate human model of itch and, in combination with cowhage, very useful to explore non-histaminergic itch pathway. The studies with doxepin and EMLA showed different effects on histaminergic and non-histaminergic itch and improved the understanding of these two pathways, necessary for the development of optimal treatments for itch.

DANSK RESUME

Kronisk kløe er et symptom i mange dermatologiske sygdomme (atopisk dermatitis, psoriasis og urticaria), men kan også ses i non-kutane tilstande (kolestase, nyreinsufficiens og perifer neuropati). Kløe påvirker i høj grad livskvaliteten for patienterne. Faktisk påvirker det vigtige funktion såsom koncentration, søvnkvalitet, seksuel aktivitet, etc., og er kendt for at øge depression og angstsymptomer. Indtil nu er effektive og kurerende behandlinger udelukket grundet den manglende viden om mulige interaktion mellem kløe og smerte.

Kløe og smerte har mange ligheder i form af neuronale mediatorer, receptorer og mønstre af neuronal sensibilisering, men overlappet mellem disse to typer af føling skal forstås bedre. De kutane pruriceptive underpopulationer der transmitterer kløe er en del af en større population af fibre der også responderer til smertefulde stimuli. Kløe kan inddeles i to forskellige systemer. Non-histamin kløe er specielt vigtig fordi det er den sværeste at behandle og er ikke-responsiv til antihistaminer. I menneskelige surrogatmodeller af kløe bruges to molekyler til at aktivere de to systemer: Histamin og cowhage. Disse modeller er brugbare til at undersøge karakteristika omkring kløe og søge nye mål for behandling, trods modellernes begrænsninger. En anden model af non-histamin kløe involverer påførelse af såkaldt bovine adrenal medulla (BAM)8-22. BAM8-22 og cowhage inducerer en histamin-uafhængig kløe ved at binde til to forskellige familier af receptorer på overfladen af de samme fibre.

Der findes adskillelige behandlinger mod kløe og de er målrettet forskellige aspekter ved kløetransmissionen. Én af disse er doxepin creme, en tricyklisk antidepressiv med stærke anti-histamin egenskaber. En anden er EMLA cremen, som er et lokalt bedøvelsesmiddel der blokerer natrium kanaler.

Baseret på dette er målene med dette PhD projekt at:

- Måle egenskaberne af en model af non-histamin kløe baseret på BAM8-22;
- Sammenligne effekten af doxepin creme på histamin og non-histamin kløe induceret af histamin, cowhage og BAM8-22;
- Undersøge effekten af gentagen påførelse af EMLA creme på neurogen inflammation, mekanisk- og temperaturfølsomhed og histamin og non-histamin kløe induceret af histamin og cowhage.

Det første studie bekræftede at BAM8-22 er en god model for non-histamin kløe uden at inducere smerte. Dette er relevant da BAM8-22 og cowhage dermed viser forskellige egenskaber, hvor cowhage inducerer smertefølelse. Det andet studie sammenlignede kløe induceret af histamin, BAM8-22 og cowhage alene og ved forbehandling med doxepin creme. Et interessant fund var at doxepin ikke kun modvirkede histamin-kløe men også reducerede non-histamin kløe. I det sidste studie

blev forskellige aspekter ved EMLA cremen undersøgt. EMLA cremen reducerede mekanisk- og temperaufølsomheden uden en dosis-kumulativ effekt. Derudover reducerede EMLA non-histamin kløe uden at påvirke histaminsystemet.

Som konklusion, er BAM8-22 en valid human surrogatmodel for kløe og, i kombination med cowhage, meget brugbar til at undersøge non-histamin kløe. Studierne med doxepin og EMLA viste forskellige effekter på histamin- og non-histamin kløe og forbedrede vores forståelse af de to kløesystemer, nødvendig for udvikling af optimale behandlinger af kløe. Danish.

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"You can never be overdressed or overeducated"

Oscar Wilde

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CHAPTER 1. INTRODUCTION

1.1. GENERAL ASPECTS OF ITCH

Itch or pruritus is defined as “an uncomfortable sensation that evokes the desire to scratch” ¹. Most people have experienced occasional pruritus, for example after a mosquito bite, wearing an unpleasant woolen sweater, after contact with toxic plants such as nettle, or an innocuous rash, etc.. In all these cases, itch lasts for a very limited period, such that most people consider pruritus a nuisance, or a minor disease ². Unfortunately, itch may persist for longer, and when it occurs for more than 6 weeks it is defined as chronic itch ². Chronic itch is a symptom of several pathological conditions, including psoriasis, atopic dermatitis (AD), cholestatic pruritus, drug-induced itch, etc., and severely impacts the quality of life of patients.

1.1.1. EPIDEMIOLOGY OF CHRONIC ITCH

Assessing the prevalence of itch is quite difficult, partly due to differing methodologies (definitions and surveys) and partly due to the heterogeneity of populations recruited in the studies ³. Prevalence could be evaluated as point (symptom experienced at the moment of the study), 12-month (symptoms experienced within a year), and lifetime prevalence (symptom experienced in any period of the life) ⁴.

In 2004, a prevalence of acute itch of 8.4% was reported in a population of 40,888 adults in Oslo ⁵. In 2007, in a cross-sectional study on Norwegian adults (18,770 subjects self-reported health with chronic itch and pain), prevalences of 20% for chronic pain and 39% for chronic itch were reported, with a high association of chronic pain with chronic itch ⁶. In 2010 and 2011, two studies were conducted in Germany. The first one was a cross-sectional study and a point prevalence of 16.8% of chronic pruritus in a working population of 11,730 was extracted ⁷. The second was a population-based cohort study with 2,540 participants and prevalences of chronic itch were calculated: point prevalence of 13.5%, 12-month prevalence of 16.4%, and lifetime prevalence of 22.0% ⁸. This year a study with 44,689 participants from 27 countries (24 EU countries, plus Norway, Switzerland, and the United Kingdom) was published and the 12-month prevalence of at least one dermatological condition or disease was 43.35% ⁹. Considering these numbers, the authors estimated that more than 94 million people in Europe experience uncomfortable skin sensations (such as itch, burning, or dryness) ⁹.

Chronic itch in children has an overall prevalence of 15%, with a country variability, and atopic dermatitis is the most common cause ³. The intensity of chronic itch increases with age ¹⁰, but older adults (59-60 years old) compared to young adults (30-45 years old) reported chronic pain more frequently than chronic itch ¹¹. The causes are dermatological disorders (psoriasis, eczema, urticaria, lichen planus, etc.), systemic conditions (diabetes, renal or hepatic issues, etc.), neurodegenerative conditions, and polypharmacy ³. In general, women report more chronic itch than men (11.9% and 9.6%, respectively) ¹². Moreover, ethnic genetic polymorphisms, different skin types, and socioeconomic factors may be involved in itch prevalence ¹³.

1.1.2. IMPACT OF CHRONIC ITCH ON QUALITY OF LIFE

Itch can be evaluated focusing on its intensity or in relation to its impact on quality of life. Several instruments were developed to this end. Health-related quality of life (HRQoL) is a multi-dimensional construct that contains several domains (such as physical and psychological health, and social functioning). The World Health Organization (WHO) developed in 1995 the WHOQOL to evaluate the impact of disease on the quality of life ¹⁴⁻¹⁸. The WHOQOL consists of six domains: physical, psychological, level of independence, social relationships, environmental health, and spirituality. Dermatology Life Quality Index (DLQI) ¹⁹ and Skindex ^{20,21} measure the skin disease impact on HRQoL, but they are not specific to itch conditions. More specific instruments to measure the itch impact are the 5-D Pruritus Scale, the Questionnaire for the Assessment of Pruritus, the ItchyQoL, and the PROMIS (Patient-Reported Outcomes Measurement Information System).

The 5-D Pruritus Scale evaluates duration, degree, direction, distribution, and disability without covering emotional involvement ²². The Questionnaire for the Assessment of Pruritus includes a verbal description of itch, affective dimensions, and severity of itch ²³. The ItchyQoL is an in-depth interview with 22 items ²⁴. The PROMIS was developed by the National Institute of Health and is based on Item Response theory; in this way, fewer items with higher precision are administrated ²⁵.

Several studies demonstrated that itch has a negative impact on quality of life ^{26,27} and increments the risk of suicidal ideation ^{28,29}. For example, a study from 2007 assessed 492 dermatology patients, and it was found that 50% of them suffer itch and fatigue, and 25% experience severe symptoms that are associated with worse skin disease and worse quality of life ³⁰. Many studies were conducted to assess the QoL in AD patients and it was found that these patients are more depressed, agitated and anxious, less energetic, and with difficulties in concentration ^{31,32}. In general, chronic itch increases stress, anxiety, and these mood disorders in turn exacerbate itch, worsening the condition ³³.

1.2. ITCH AND PAIN SIMILARITIES

Itch and pain share many similarities in terms of mechanisms and molecules involved. Both transmissions involve the C-fibers, sensory nerves, and dorsal horn, and at the cerebral level, they share areas such as the thalamus, anterior cingulate and insular cortex, somatosensory cortex, and motor areas³⁴. Itch and pain can be both defined as neuropathic, neurogenic, or psychogenic, and they can be provoked at each level. There are many common mediators (endothelins, substance P, neurotensin, prostaglandins, opioid peptides, etc.) and also the activation of transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) may induce itch or pain^{1,35,36}. The inflammation process is involved in pain and itch, facilitating them. Moreover, some treatments are used in both pain and itch management (capsaicin, cannabinoids, gabapentin, anticonvulsant drugs, antidepressants, etc.)³⁴.

1.2.1. DYSESTHESIAS

Dysesthesia is defined as “an unpleasant abnormal sensation”³⁷. Neuroplastic changes in nociceptive and pruriceptive systems are considered the cause of dysesthesias^{38–40}. The manifestations of dysesthesias are very similar for itch and pain conditions: it is possible to observe allodynia and hyperknesis in patients affected by chronic itch disorders (e.g. atopic dermatitis or neuropathic itch)³⁸ as well as allodynia and hyperalgesia in chronic pain patients (e.g. affected by neuropathic pain)^{41,42}. The terms allodynia and allodynia refer to an itch or pain sensation respectively in response to an innocuous stimulus^{43–45}. On the other hand, hyperknesis and hyperalgesia are states in which increased itch or pain are felt after an itchy or painful stimulation respectively^{39,43,46}. For all these sensory phenomena, it is possible to distinguish between “primary” and “secondary”. They are considered “primary” when the altered sensation occurs within a lesional skin or, in the research field, in the noxious or pruritic stimulus application area, and “secondary” if the sensation occurs in the surrounding area^{40,47}.

The response evoked mechanically by thin filaments or weighted needles is a pricking sensation associated with a delayed mild itch or tickling^{48–50}, but if the stimulus is dynamic or less intense, the response is often described as tickling associated with motor responses such as scratching and rubbing (in accordance with itch definition)⁵¹.

In 1922, von Frey suggested that superficial tickling could be a mixture of tactile and itch impulses⁵². A few years later, Pritchard conducted studies on mechanically induced itch in patients with neuropathic lesions. No itch reduction was found in patients with selective touch hypoesthesia and, independently of touch sensation, itch

and pain were always altered in parallel^{53–55}. In 1938, Bickford assessed mechanically evoked itch after histamine application, and the sensation evoked in the surrounding skin area was called “itchy skin”⁵⁶. In the late 1980s LaMotte et al. extended these experiments and the terms “alloknesis” and “hyperknesis” replaced “itchy skin”, owing to an improved knowledge of somatosensory neurophysiology^{48,56,57}.

These terms are now mostly used in relation to mechanical stimulations, but not exclusively. Recently the existence of so-called “warmth alloknesis” was demonstrated, being an itch sensation elicited by innocuous warmth stimuli observed in patients with chronic itch conditions^{58,59}. Moreover, increased itch response to a chemical itch provocation could be defined as hyperknesis.

The dysesthesias may last few minutes or hours after itch provocation or may be persistent, for example in AD patients^{43,60,61}.

1.2.2. SENSORY SENSITIZATION

The International Association for the Study of Pain (IASP) defined the term nociception as “the neural process of encoding noxious stimuli”, without the implication of pain sensation³⁷. Nociceptors are “high-threshold sensory receptors of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli (stimuli that are damaging or threaten damage to normal tissues)”³⁷. In primates, pruriceptors (units responsive to pruritogens) are nociceptors as well; in fact, they are responsive to noxious stimuli⁶². In humans, evidence suggested the existence of “pruriceptive nociceptors” and “non-pruriceptive nociceptors”⁶².

Pruriceptors and nociceptors can increase the sensitivity and the responsiveness to a variety of stimuli, and this event is called sensitization^{63–65}. Studies in chronic pain patients suggest the involvement of sensitization in the chronification of pain and dysesthesias^{63,66}. In the itch scenario, the neuronal sensitization could be involved in itch chronification and this hypothesis is proved by different evidence. First, C-fibers are characterized by tachyphylaxis (decrease responsiveness), and the chronic spontaneous itch in e.g. inflammatory dermatoses could be due to the continuous release of endogenous pruritogens^{67–69}. Second, itch and pain dysesthesias share some similarities regarding the spatiotemporal properties^{48,49}. Moreover, to support the hypothesis, several events are involved, for instance the antipruritic effect of centrally acting noradrenergic, serotonergic, and GABAergic drugs⁷⁰, the absence of a strong correlation between itch and lesional severity^{71,72}, changes in the expression of molecular transducers on epidermal C-fibers⁷³. However, pain sensitization has been deeply studied^{63,66,74,75}, while itch sensitivity was poorly investigated^{38,76}; however, circumstantial evidence^{40,77} suggested that nociceptive and pruriceptive sensitizations

share mechanisms and the discriminant between the two sensitizations is still unknown.

The nociceptive and pruriceptive fibers' sensitization occurs at peripheral and central levels in the nervous system^{40,60,78}.

1.2.2.1 Peripheral sensitization

Peripheral sensitization is defined as an “increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields”³⁷. A local inflammation (characterized by redness, swelling, a sensation of warmth, and pain) often causes peripheral sensitization, even though there are peripheral neuropathic conditions without inflammation⁶². Cutaneous inflammation involves different cells such as immune cells, keratinocytes, endothelial cells, and primary sensory afferent neurons⁶². There are different causes of the inflammatory responses, like sunburn, contact with irritant substances, or a noxious heat stimulus; the inflammatory reactions in these cases are mechanistically distinct, with differences in sensory aberrations and temporal development^{79,80}. The mediators involved are e.g. cytokines (interleukins and chemokines), nerve growth factor (NGF), tumor necrosis factor alpha (TNF α), prostaglandins released by the immune cells and keratinocytes, neuropeptides (substance P, and calcitonin gene-related peptide (CGRP)) from the local peptidergic fibers^{65,81–83}. These molecules are not only involved in inflammation, but also in the acute and prolonged development of mechanical and thermal hyperalgesia, edema, and extravasation^{65,81,84}. Moreover, many of these mediators are directly or indirectly involved in pain and itch signaling and peripheral sensitization^{85,86}. Specific chemokines and interleukins (e.g., CCL17, IL-13, and IL-31) sensitize C-nociceptors in several itch conditions associated with inflammation (for example AD)^{87,88}: experiments conducted in rodents revealed the TRPA1 upregulation induced by IL-13⁸⁸.

Lastly, many intracellular signaling pathways in nociceptive neurons are sparked by inflammation, and phosphorylation and transcription of nociceptors' transduction molecules (e.g. TRPs and sodium channels) are increased with the consequent increase of excitability of nociceptive and pruriceptive A δ - and C-fibers^{65,89}.

1.2.2.2 Central sensitization

Central sensitization is an “increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input”³⁷. The mechanisms involved are spinal disinhibition, activation of glial cells, synaptic

plasticity, and altered descending modulation^{63,66,90}. Specific mechanisms and signaling molecules affect central transmission by facilitating or modulating excitatory synaptic communication between first- and second-order neurons in the spinal dorsal horn; these mechanisms and molecules are, for example, glutaminergic mechanisms, CGRP, and brain-derived neurotrophic factor^{63,74}. Increased sensitivity to noxious stimuli can be due to spinal and supraspinal alterations such as an increase in the glial activity, long-term potentiation of synapses, and hyper-responsiveness of nociceptive spinal dorsal horn neurons^{63,91}. The increased responsiveness of nociceptive and pruriceptive neurons located in the spinothalamic tract to normal or low-threshold input from primary neurons may explain pain and itch dysesthesias^{38,40,51,63,74}.

1.3. ITCH CODING HYPOTHESIS

Itch- and pain-evoking stimuli activate overlapping nociceptors populations⁶⁰, spinal neurons⁹², and ascending sensory pathways^{83,91,93}. Nevertheless, the sensations evoked by pruritic and algesic stimuli are different, as well as the nocifensive responses (scratching and biting for itch, wiping, and withdrawal for pain)⁹⁴. The implication is that there is a certain central specificity for itch and pain.

Several theories were developed for itch coding and the discrimination between itch and pain: intensity, specificity, selectivity, and spinal-contrast hypothesis.

1.3.1. INTENSITY HYPOTHESIS

The intensity hypothesis posits the existence of only one type of nociceptors responsive to both itch and pain. The discrimination between itch and pain sensation is then possible by different encoding in the neurons firing rates (low firing rate for itch, higher for pain)⁹⁵.

This hypothesis was proposed by von Frey and he suggested that mildly painful stimuli elicit itch⁵². Circumstantial evidence support this hypothesis: unmyelinated peripheral fibers carry both itch and pain sensations, itch elicited by mild punctuate mechanical stimuli, and the contralateral ablation of itch and pain after an anterolateral ascending tract cordotomy.

Against this hypothesis, the less intense pain, and no itch, evoked by low concentrations of algogens, experiments of stimulation of afferent nerve fibers in which a lower stimulation frequency resulted in less itch or pain sensation, but not in

a switch from pain to itch ^{96,97}, and the fact that opioids reduce pain and cause or aggravate itch ³⁵.

1.3.2. SPECIFICITY HYPOTHESIS

The specificity hypothesis postulates the existence of two parallel pathways for itch and pain: distinct primary afferents are selectively involved in itch or pain transmission (pruriceptors and nociceptors respectively) and connect central pathway dedicated to itch or pain sensation.

This hypothesis is supported by the existence of a subpopulation of mechano-insensitive C-fibers (CMi-fibers) with very low conduction velocities that specifically mediates itch ^{98,99}. Contrary to this hypothesis, the same CMi-fibers also respond to several algogens causing subjective pain ^{100,101}.

1.3.3. SELECTIVITY HYPOTHESIS

The selectivity hypothesis is similar to the specificity hypothesis, but it includes an inhibitory modulation of itch by nociceptive input via interneurons. In other words, itch is perceived when there is a predominant activation of itch-selective fibers. When there is a co-activation of pain- and itch-selective fibers, pain is perceived ^{100,102,103}.

Studies in patients with chronic itch support this hypothesis: it was demonstrated that in these patients, noxious stimuli (mechanical, thermal, and chemical) induced itch rather than pain ^{60,61}.

1.3.4. SPATIAL-CONTRAST HYPOTHESIS

In the 1990s, the spatial-contrast hypothesis was formulated ^{104,105} and it suggests that itch is evoked if c-nociceptors are activated in a scattered spatial pattern ^{106,107}. In other words, if only a few nociceptive fibers are activated and the neighboring units are silent, itch is perceived, while, if more units in the same skin area are activated, pain is felt.

To support this hypothesis the fact that polymodal C-fibers (PmC-fibers), responsive to cowhage, are not itch-selective and their activation may result in itch or pain sensation ^{67,106,107}. Moreover, in mice studies, it was estimated the approximate amount of subpopulation of C-fibers: histamine-sensitive C-fibers represent 5-10%,

pruriceptive C-fibers expressing Mas-related G-protein coupled receptor A1 (MRGPR A1) are 5% of all C-fibers, while in the DRG population the chloroquine-responsive fibers represent 12.8%^{98,100,108,109}. The distribution of these fibers creates a spatial contrast signaling pattern.

1.4. HISTAMINERGIC AND NON-HISTAMINERGIC ITCH

Itch can be divided into two categories focusing on the peripheral pathways involved: histaminergic and non-histaminergic itch. Two different classes of C-fibers are involved, mechano-insensitive (CMi) and mechano-heat sensitive “polymodal” (PmC) C-fibers^{110–115}. These subgroups of fibers differs also in conduction velocity, size of receptive fields, and the degree to which they show activity-dependent slowing^{113,116,117}.

1.4.1. HISTAMINERGIC ITCH

During the last century, histamine-induced reactions on the skin started to be investigated. Lewis analyzed skin responses¹¹⁸, while Bickford described the “itchy skin” phenomenon (later described as dysesthesias, as previously mentioned) after histamine application⁵⁶. Cormia and Kuykendall, by performing histamine injections, conducted psychophysical studies and they found lower itch threshold and hyperknesis in lesioned skin of itch patients^{119,120}. Keele and Armstrong evaluated the effect of different concentrations of histamine applied on exposed dermo-epidermal nerves. They found that a low concentration induced itch, while pain was evoked with an higher concentration, as well as deeper histamine injections^{121,122}.

After the microneurography technique development, a study proved a strong correspondence between subjective itch perceived after histamine iontophoresis and activation of specific CMi-fibers^{98,99}. The C-fibers responsive to histamine represent 5% of all skin nociceptors and are just a minority of all C-fibers usually mechanosensitive and histamine-insensitive^{123,124}. The subpopulation of CMi-fibers responsive to histamine is mechanical insensitive, with a low conduction velocity, large innervation territories, and high transcutaneous electrical thresholds. Moreover, when histamine is applied and the CMi-fibers are activated, also a generation of an axon reflex flare occurs. The CMi-fibers resulted also sensitive to various algogens (such as bradykinin and capsaicin) causing spontaneous pain^{100,101}. In patients with chronic itch, a microneurography study proved the spontaneous activity of these fibers¹²⁴.

The present knowledge of histaminergic itch is that it is transmitted through CMi-fibers expressing histamine-receptors 1 and 4 (H1/4R) and TRPV1 on their surface^{125,126}.

1.4.2. NON-HISTAMINERGIC ITCH

Histamine represents a golden mediator for the study of itch, but histaminergic itch cannot account for the itch present in several clinical conditions^{86,127–129}. For example, the treatment with antihistamines does not ameliorate itch in patients with AD, which did not result different from healthy subjects in sensitivity to extra-lesional histamine provocation^{127,128,130,131}.

Shelly and Arthur observed, in the 1950s, a kind of itch distinct from histamine-induced itch, which could be induced by cowhage^{132–134}. This cowhage-induced itch did not produce any flare reactions and it was more similar to the itch present in certain pathological conditions¹³⁵. The extraction of the active itch-inducing enzyme (mucunain) from cowhage spicules was performed by Shelly and Arthur^{132,134}. Reddy et al., in 2008 demonstrated that mucunain is a ligand of proteinase-activated receptors 2 and 4 (PAR2/4) expressed on epidermal C-fibers¹³⁶. Studies in recent years proved that itch induced by cowhage is almost exclusively transmitted by PmC-fibers^{107,137,138}. This pathway of itch was called non-histaminergic itch, in contrast with histaminergic itch^{137,139,140}. These definitions are not optimal: histaminergic itch refers to the neuronal pathway that can also be activated by other compounds without the binding to histamine receptors^{86,141}, while non-histaminergic itch is only a definition by negation. Moreover, in rodent experiments, CMi-fibers activated by histamine and PmC-fibers activated by cowhage resulted not so clearly separated¹⁴² and further human studies are needed to elucidate these mechanisms¹⁴³. Lastly, PmC-fibers respond not only to cowhage but also to other substances (like BAM8-22, trans-cinnamaldehyde, etc.¹⁴⁰) and pain-evoking stimuli^{107,138,144}.

1.5. HUMAN EXPERIMENTAL SURROGATE ITCH MODELS

A human experimental surrogate model aims to temporally reproduce specific symptoms and mechanisms of a disease^{63,145–148}. For example, to mimic the cold allodynia observed in chemotherapy-induced peripheral neuropathy, high concentration of L-menthol is used, while topical capsaicin mimics cutaneous heat and pinprick hyperalgesia of post-herpetic neuralgia^{41,149–152}. The study of a disease or a clinical condition can be conducted through three different kinds of research in humans:

- 1) Basic mechanistic studies in healthy subjects;
- 2) Proof-of-concept studies to assess the efficacy of new or existing drugs or therapies;
- 3) Clinical studies to evaluate gain and/or loss of function in patients compared to healthy control.

For ethical reasons, human surrogate models of itch can only be acute or subacute without the induction of the prolonged skin inflammatory state that is present in chronic itch conditions^{145,147}. Up to now, human models of itch involve the use of various chemicals¹⁵³. One problem is the impossibility of quickly switching on/off the itch sensation provoked by the chemicals. On the contrary, this could be obtained by the use of artificial stimuli (such as electrical) like the ones used as pain models, but these stimuli do not exhibit solid results¹⁵³. As previously mentioned, nociception and pruriception share some similarities and it is confirmed also by the fact that commonly used algogens (eg. capsaicin, bradykinin, trans-cinnamaldehyde, etc.) can induce both itch and pain depending on the administration circumstances^{154,155}, as well chemical pruritogens can also elicit mild pain^{61,156,157}.

1.5.1. HISTAMINE

Histamine is the golden model of itch in human studies¹⁴¹. Histamine was available from the early 20th century, after its discovery by Henry Dale in 1910¹⁵⁸. Lewis started to use histamine in human studies to examine the cutaneous capillary reactions, followed by Bickford, who studied histamine-induced itch and sensitivity changes^{56,158,159}. Histamine elicits itch sensation (5 to 20 minutes) with occasional stinging pain, mild burning, and warmth sensation^{155,156}. Histamine also causes a non-neuronal microedema (wheal) and an extent neurogenic flare (axon-reflex flare)¹⁵³. Microneurography studies demonstrated that histamine activates mechano-insensitive C-fibers^{100,160}. Histamine (usually dihydrochloride salt at a 0.1% to 1% solution) can be applied through skin prick test lancets, intradermal injections, abrasion, inactivated cowhage spicules, or iontophoresis¹⁴¹. The deepness of histamine stimulation seems to be related to the pain sensation in addition to itch, in fact, histamine injections are associated with pain while a more superficial application mostly causes itch^{121,122,161}. Regarding the itch coding hypothesis (previously described in chapter 1.3), the predominant itch sensation evoked when histamine is applied dermo-epidermally could be explained by the specificity theory and it could be assumed that CMi-fibers are itch selective. On the other hand, the low density of histamine-sensitive fibers (5 to 10% of the C-nociceptor population) could be also related to the spatial-contrast hypothesis due to the impossibility of activation of a high amount of fiber in a given skin area¹⁰⁶.

Histamine-induced itch can be abolished using antihistamines. In urticaria, the treatment with antihistamines is very effective in itch relieving¹⁵³, and histamine could be considered a good model to mimic the pathological condition. However, most clinical chronic itch conditions are unresponsive to antihistamine^{86,141}. Especially in atopic dermatitis (AD), it was demonstrated the inefficacy of the treatment⁸⁵, but it is also true for neuropathic, dermatologic, and systemic itch conditions¹⁵³.

1.5.2. COWHAGE

Cowhage refers to the spicules present on the pod of *mucuna pruriens*, legume native in tropical Africa and Asia. In the 1950s, Shelley and Arthur started their experiment with cowhage. They extracted the enzyme mucunain and demonstrated that cowhage-induced itch is different from histamine-evoked itch^{132,134}. The differences in sensory qualities, the absence of flare, and the higher sensitization to cowhage compared to histamine in AD patients indicated cowhage as a better itch model for this pathology^{43,162}. Cowhage, mostly applied through spicules, evokes a stronger itch than histamine but with a higher pain component (e.g., stinging, pricking, and burning sensation)^{156,157,163}. Cowhage-induced itch quickly increases, peaks around 1-2 minutes, and decreases faster than histamine-induced itch (5-15 minutes)¹⁵³.

Mucunain evokes itch by binding to proteinase-activated receptors 2/4 expressed on epidermal C-fibers¹³⁶. In a recent study, the involvement of Mas-related G protein-coupled receptors MrgprX1/2 was also proposed¹⁶⁴. Mechanistic studies in human and nonhuman primates demonstrated that itch induced by cowhage is mostly transmitted by mechano-heat sensitive “polymodal” C-fibers (PmC-fibers)^{107,137}. These fibers are also responsive to various pain stimuli, underlying the lack of itch-specificity of these fibers^{107,137}. cowhage-induced itch can probably be better explained by the spatial-contrast hypothesis due to the area provoked by the spicules. The absence of a neurogenic response induced by cowhage is in line with previous knowledge that only CMi-fibers induce an axon-reflex flare¹¹⁷.

Lastly, itch induced by cowhage is refractory to treatment with antihistamines¹³⁷, electing cowhage as a better model to mimic several clinical itch conditions.

1.5.3. BAM8-22

Bovine adrenal medulla (BAM)8-22 is an endogenous peptide derived from the hormone proenkephalin A. It is an agonist of MrgprX1 and, via the Gαq/11 pathway, induces itch^{165,166}. The MrgprX1 receptors are expressed on mechanosensitive C-

fibers which terminate in the superficial layers of the skin ¹⁶⁷. Sikand et al. applied on the skin of healthy volunteers a BAM8-22 solution (concentration up to 4 mg/ml) through inactivated cowhage spicules ¹⁶⁸. It was found that BAM8-22 evokes an itch intensity similar to histamine (10 mg/ml applied in the same way), but the itch sensation is accompanied by a pricking/stinging sensation and the ratio between itch and pain is on par with cowhage ¹⁶⁸. They also demonstrated that antihistamines do not affect BAM8-22-induced itch, defining it as a non-histaminergic itch model ¹⁶⁸. Itch is elicited also through intradermal injections of BAM8-22 in sterile extracellular fluid ^{40,168}. The intensity and the temporal profile of itch are similar to histamine, nevertheless, the pain sensation is higher ^{40,168}. The BAM8-22 properties as a pruritogen will be deeper discussed in chapter 3.

1.5.4. OTHERS

1.5.4.1 Capsaicin

Capsaicin is the active compound of chili peppers and it is an agonist of transient receptor potential (TRP) cation channel vanilloid-1 (V1) ¹⁶⁹. Through the binding of TRPV1 receptors, capsaicin activates a subgroup of C- and A δ -nociceptors ^{169,170}. Capsaicin is a well-established algogen, but it also elicits itch when applied in certain conditions. The topical application (cream or dermal solution) of low-concentration of capsaicin (0.025 to 0.075%) induces both itch and pain ^{171–173}, while higher concentrations cause mostly pain ^{174,175}. A highly localized capsaicin administration (e.g. by skin prick lancet or inactivated cowhage spicules) induces mostly itch or the same intensity of itch and pain ^{176,177}, and this seems to validate the spatial-contrast hypothesis. Capsaicin represents a good model of mixed itch and pain sensations ¹⁵⁴, but it is not a valid pruritic model due to the absence of chronic itch conditions associated with the TRPV1 signaling and/or heat hyperalgesia ^{43,86}. The block of TRPV1 channels, using a high concentration of capsaicin (patch 8%), has a profound impact on histamine- and cowhage-induced itch ¹⁷⁴.

1.5.4.2 TRPA1 agonists

TRPA1 agonists are not largely used as itch models, even though TRPA1 is considered a downstream mediator in itch pathways involving the activation of several pruritogen-receptors ^{163,178}. TRPA1 is probably expressed by cutaneous mechano-insensitive as well as mechanosensitive C-fibers ^{179,180}. One problem of human models involving TRPA1 agonists is the irritant contact sensitization they act on ¹⁸¹. One TRPA1 agonist is the trans-cinnamaldehyde (CA). The application of 5% CA solution induces a predominant itch sensation accompanied by burning and warmth in some

subjects¹⁸², while the nociceptive sensation raises by increasing the concentration of CA^{183,184}. With the 5% solution, alloknesis and hyperknesis are poorly observed¹⁸². This absence and the presence of pain represent a limit of the CA model of itch. Another TRPA1 agonist is mustard oil (allyl isothiocyanate). In rodent and human studies, mustard oil applied as a transdermal solution is an exclusive pain model, while no studies were conducted with a low concentration of it or with a highly localized application¹⁵³.

1.5.4.3 Mas-related G protein-coupled receptor agonists

Chloroquine is a MrgprA3 agonist and induces itch in rodents activating a subgroup of C-fibers selective for itch^{108,185}. In humans, chloroquine is used as an antimalarial drug. It was reported severe pruritus elicited by the treatment with chloroquine in a black African cohort with malaria¹⁸⁶. Up to now, a human itch model based on chloroquine is missing. B-alanine is an MrgprD receptors agonist. In human models, β -alanine can be administrated by skin prick lancet, intradermal injection, or orally^{187,188}. It induces a less intense and persistent itch than histamine or cowhage¹⁸⁸, not enough to induce any itch sensitization¹⁸⁹, making β -alanine insufficient as an itch-inducer in human models.

1.5.4.4 Serotonin

Serotonin is a neurotransmitter involved in the inflammatory process. It was demonstrated by microneurography studies that serotonin activates both mechanosensitive C-fibers and histamine-responsive mechano-insensitive C-fibers¹⁰⁰. In humans, serotonin generally only elicits mild itch (approx. half of histamine-induced itch)^{61,190}, even though, in rodents, induces a scratching behavior when injected intradermally¹⁹¹. Serotonin-induced itch is not abolished by antihistamines^{61,153}, but it is not a used model due to the weak itch induction.

1.5.4.5 Bradykinin

Bradykinin, an endogenous nociceptor released during inflammation^{68,192}, is a low-specific activator of C-fibers¹⁰⁰. Its application through cutaneous microdialysis induces weak burning pain¹⁰⁰, while mild itch and pain are reported when bradykinin is introduced by iontophoresis⁶¹. In patients with AD, bradykinin induces a more robust itch, underlying peripheral sensitization⁶¹. In general, pain is more robust than itch and for this reason, bradykinin is not a used itch model¹⁵³.

1.5.4.6 Acetylcholine

Acetylcholine is an autonomic nervous system transmitter also involved in sweat secretion ¹⁵³. AD patients complain that sweat aggravates itch and for this reason acetylcholine started to be studied as a pruritogen. It was tested in several studies, and it was found that acetylcholine induces more pain than itch and activates both mechanosensitive and mechano-insensitive C-fibers ^{100,193}, while, in AD patients, it elicits more itch than pain ³⁸. Acetylcholine is not used as an itch-inducer for the same reason as bradykinin.

1.5.4.7 Substance P

The vasoactive neuropeptide substance P is released after the stimulation of peptidergic cutaneous C-nociceptors ¹⁹⁴. It recruits immune and endothelial cells and it is probably involved in the C-nociceptor sensitization ¹⁵³. In rodents, substance P induces a histamine-independent scratch behavior ¹⁹⁵, while in humans substance P-induced itch seems to be dependent on histaminergic pathway ^{61,190}. In any case, substance P causes a less robust itch than histamine, but the same cutaneous pain in humans ^{61,190}.

1.6. THERAPIES OF PRURITUS

In the past few decades, the knowledge about itch substantially improved, but despite this, the treatment options remain sub-optimal. One of the reasons could be the difficulty and/or impossibility to identify the underlying etiology of itch ². Up to now, the approach to itch management is individually tailored and a precise history and physical evaluation are essential for identifying a possible underlying cause and then choosing a therapy ². The treatment of the underlying causes or disease usually improves also the itch conditions. In general, if itch is mildly perceived or is localized, topical interventions are adopted; if pruritus is severe or generalized, systemic treatments are utilized ².

Most of the therapies act on three main families of receptors present in the cutaneous C-fibers: voltage-gated sodium (Nav) channels, G protein-coupled receptors (GPCRs), and transient receptor potential (TRP) channels ¹⁹⁶. Nav channels are involved in the generation of action potentials in the nervous system. One out of the nine existing subtypes is involved in itch transmission: Nav1.7 (expressed in peripheral nerves and spinal cord) ¹⁹⁷. GPCRs are present on keratinocytes, primary sensory neurons (at the nerve endings), and second-order neurons in the spinal cord ¹⁹⁶. This family of receptors includes histamine, cannabinoid, and opioid receptors ¹⁹⁸.

TRPs are cation channels and they can be downstream of GPCRs, increasing the propagation of the signal ¹⁹⁶. There are several subfamilies and three are particularly relevant in itch: TRP vanilloid (eg, TRPV1), TRP ankyrin (eg, TRPA1), and TRP melastatin (eg, TRMP8). TRPs are also directly activated by different stimuli depending on the subfamily; the stimuli are, for example, substances (capsaicin, menthol, mustard oil), changes in temperature, pH, mechanical stimuli, etc. ¹⁹⁸.

1.6.1. TOPICAL TREATMENTS

| Therapy | Mechanism | Use in clinic |
|---|--|---|
| Moisturizers, emollients, and barrier creams | Action on the skin barrier | Nocturnal itch Dry skin Atopic skin |
| Topical antihistamines | Block of histamine receptors | AD Contact and nummular dermatitis Lichen simplex chronicus |
| Local anesthetics | Block of sodium channels | Neuropathic pruritus Facial and anogenital itch Pruritus in hemodialysis patients Itch in chronic cholestatic liver diseases |
| Topical corticosteroids | Reduction of skin inflammation | Inflammatory skin disorders (e.g. AD) |
| Topical immunomodulators | Anti-inflammatory action | AD Prurigo nodularis Lichen sclerosis Anogenital pruritus Chronic irritative hand dermatitis |
| Menthol | Cooling properties through the binding of TRPM8 | Useful in patients who have itch relief with cooling |
| Capsaicin | TRPV1 agonist | Neuropathic itch conditions Aquagenic pruritus Prurigo nodularis CKD-associated pruritus |
| Topical Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) | Block of the production of prostaglandins and thromboxane by the inhibition of the enzyme cyclooxygenase | Prurigo nodularis Postherpetic neuralgia Lichen simplex chronicus |
| Topical cannabinoids | Cannabinoid receptors CB1 and CB2 agonists | AD Prurigo nodularis Lichen simplex CKD-associated pruritus |

Table 1: Topical treatments of itch, their mechanisms of action, and their clinical uses.

1.6.1.1 Moisturizers, emollients, and barrier creams

Moisturizers, emollients, and barrier creams are the simplest itch treatment and act on the skin barrier ². In AD patients an association was observed between the itch intensity and the transepidermal water loss (TEWL) that reflects the barrier function ¹⁹⁹. In particular, TEWL is higher during the night and for this reason, these creams may be useful for nocturnal itch and dry or atopic skin ^{200,201}. Moreover, creams with a low pH help in the preservation of the normal pH of the skin surface ameliorating the barrier function ². An acid pH may also be useful to reduce the activity of serine proteases, and endogenous PAR2 agonists ^{73,202}.

1.6.1.2 Topical antihistamines

Histamine is a mediator of itch and histamine receptors 1 and 4 (HR1 and 4) are involved in itch mechanisms ¹⁹⁶. After the binding of histamine to HR1, there is an increase of intracellular calcium via phospholipase C. Moreover, TRPV1 receptors are involved ²⁰³.

Doxepin is a tricyclic antidepressant and an HR1 and 3 antagonist ², for this reason, it is used in clinic as antihistaminergic cream. It was demonstrated that doxepin 5% ameliorates the itch condition of several diseases such as AD, contact and nummular dermatitis, and lichen simplex chronicus ^{204,205}. Its side effects include drowsiness, allergic contact dermatitis, and localized cutaneous burning ^{204,205}. The doxepin mechanism of action and its effect on histaminergic and non-histaminergic itch will be discussed more deeply in chapter 4.

1.6.1.3 Local anesthetics

Local anesthetics are antagonists of sodium channels with a consequent block of nerve conduction ¹⁹⁶. Pramoxine 1%, lidocaine 5%, and the eutectic mixture of lidocaine 2.5% and prilocaine 2.5% (EMLA) are common topical local anesthetics ^{2,206–208}. They often represent the first-line agents in the management of itch (particularly neuropathic pruritus, facial, and anogenital itch ²⁰⁹) due to their minimal risk and low costs ²¹⁰. It was demonstrated that pramoxine reduces pruritus in adult hemodialysis patients ²¹¹ and intravenous lidocaine is efficient against itch in patients with chronic cholestatic liver diseases ²¹². In any case, local anesthetics have different effects between patients, only short-term benefits, and can only be used in skin diseases (e.g. AD) ^{196,213}. The mechanism of action of EMLA cream and its effect on histaminergic and non-histaminergic itch will be covered in detail in chapter 5.

1.6.1.4 Topical corticosteroids

The reduction of skin inflammation by topical corticosteroids is considered the cause of their antipruritic effect ². They are used in clinic to treat itch in inflammatory skin disorders (e.g. AD) for a short period ². It was confirmed that hydrocortisone 2.5% reduces experimentally induced itch compared to placebo ²¹⁴. The side effects include telangiectasia (dilation of small blood vessels), skin atrophy, and hypothalamus-pituitary axis suppression ².

1.6.1.5 Topical immunomodulators

The topical calcineurin inhibitors (TCI) reduce itch probably due to their anti-inflammatory properties ². The TCI (such as tacrolimus and pimecrolimus) reduce pruritus in AD, prurigo nodularis, lichen sclerosis, anogenital pruritus, chronic irritative hand dermatitis, and graft-versus-host disease ^{215–217}. They should be used for short periods or in generalized pruritus, and transient burning and stinging sensations are usual side effects ².

1.6.1.6 Menthol

Menthol is a natural cyclic terpene alcohol from the peppermint plant and induces a cool sensation through the binding of TRPM8 receptors ^{2,196,218,219}. Thanks to its cooling properties, it is particularly useful in patients who experience itch relief with cooling ^{209,210}. Low concentrations of menthol (1 to 5%) ameliorate itch conditions, while irritation can be caused by higher doses ²⁰⁹.

1.6.1.7 Capsaicin

The antipruritic effect of capsaicin is probably due to its effect on TRPV1 receptors ¹²⁵. When capsaicin binds TRPV1, PmC-fibers are quickly depolarized with a consequent release of substance P and other neuropeptides (such as somatostatin, calcitonin gene-related peptide (CGRP), and neurokinin) generating hypersensitization, neurogenic inflammation, and desensitization of the nerves ²²⁰. Capsaicin is used in clinic for neuropathic itch conditions such as notalgia paresthetica, brachioradial pruritus, and postherpetic pruritus ²²⁰. Capsaicin also relieves pruritus in aquagenic pruritus, prurigo nodularis, and pruritus associated with chronic kidney disease (CDK) ^{221–225}. More concentrations are used, several applications of low concentration (0.025% to 0.075%) or a single high concentration

(8% patch)²¹⁰. The application of capsaicin is associated with dose-dependent burning or stinging sensations²²⁰.

1.6.1.8 Topical Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs inhibit the enzyme cyclooxygenase, and this block the production of prostaglandins and thromboxane A₂¹⁹⁷. Their action prevents a peripheral neural sensitization caused by peripheral prostaglandins, such as prostaglandin E₂²²⁶. Topical acetylsalicylic acid (aspirin) ameliorates itch in prurigo nodularis, postherpetic neuralgia, and lichen simplex chronicus^{209,210}.

1.6.1.9 Topical cannabinoids

Topical cannabinoids are agonists of cannabinoid receptors CB₁ and CB₂ present on cutaneous sensory nerve fibers, keratinocytes, and mast cells; and they reduce histamine-induced itch in humans^{227,228}. It was demonstrated that N-palmitoylethanolamine, a CB₂ agonist, has an antipruritic effect in AD, prurigo nodularis, lichen simplex, and chronic kidney disease (CKD)-associated pruritus^{2,229,230}.

1.6.2. SYSTEMIC TREATMENTS

| Therapy | Mechanism | Use in clinic |
|-------------------------------|--|---|
| Antihistamines | Block of histamine receptors 1 | Urticaria Mastocytosis |
| Antidepressants | Inhibition of the reuptake of serotonin and norepinephrine at the nerve synapse Histaminergic, muscarinic, and alpha-adrenergic receptors antagonists | Chronic urticaria Uremic pruritus Notalgia paresthetica Brachioradial pruritus CKD-associated pruritus Poststroke pruritus |
| Opioid agonist and antagonist | k-receptor agonists μ-receptor antagonists | Cholestasis AD End-stage renal disease Urticaria Inflammatory skin diseases or systemic diseases CKD-associated pruritus |
| Neuroleptics | Block of nociceptive, and thus pruriceptive, sensations to the brain | Neuropathic pruritus (brachioradial pruritus and notalgia paresthetica) CKD-associated pruritus Pruritus associated with cutaneous lymphoma |
| Immunosuppressants | Anti-inflammatory action | AD |

Table 2: Systemic treatments of itch, their mechanisms of action, and their clinical uses.

1.6.2.1 Antihistamines

Oral antihistamines are particularly efficient only against itch from urticaria and mastocytosis ^{2,196}. Moreover, the first-generation antihistamines have a sedating effect due to the poor selectivity for HR1 and their additional interaction with serotonergic, dopaminergic, muscarinic, and alpha-adrenergic receptors ¹²⁶. They can be useful in helping to improve sleep in patients with intense pruritus during the night ^{2,196}. Second-generation antihistamines are more specific for HR1 and are not sedating, so they can be used for relieving itch during the day ^{2,126}.

1.6.2.2 Antidepressants

Tricyclic antidepressants (TCAs) inhibit the reuptake of serotonin and norepinephrine at the nerve synapse, increasing neurotransmission ¹⁹⁶. They are also antagonists of histaminergic, muscarinic, and alpha-adrenergic receptors ¹⁹⁶. Doxepin and amitriptyline are two TCAs used in itch therapy. Oral doxepin is utilized in chronic urticaria and uremic pruritus ^{209,231,232}. Amitriptyline is useful in notalgia paresthetica, brachioradial pruritus, CKD-associated pruritus, and poststroke pruritus ²³¹. TCAs require continuous monitoring and dose titration to avoid side effects ²³³.

Selective serotonin reuptake inhibitors (SSRIs) inhibit the presynaptic serotonin reuptake, both peripherally and centrally ¹⁹⁶. SSRIs are used when chronic itch is refractory to other therapies, even though the mechanism is still unclear ¹⁹⁶. Paroxetine and fluvoxamine reduce pruritus in systemic lymphoma, AD, and solid tumors ^{209,231}.

Serotonin norepinephrine reuptake inhibitors (SNRIs) inhibit the reuptake of both serotonin and norepinephrine in the presynapse ¹⁹⁷. Mirtazapine has an effect on chronic and in nocturnal itch due to its sedating property ^{2,196,234}. It also may be effective on pruritus in CKD, leukemia, cholestasis, lymphoma, and atopic dermatitis ^{209,234}.

1.6.2.3 Opioid agonist and antagonist

The opioidergic system has been found to have a role in itch pathophysiology: k-opioid receptor antagonists and μ -opioid receptor agonists elicit pruritus, while k-receptor agonists and μ -receptor antagonists have the opposite effect ^{235–237}. Naltrexone and nalmeferene, two μ -receptors antagonists, show an antipruritic effect in cholestasis, atopic dermatitis, burns, end-stage renal disease, and urticaria ^{238–243}. Nevertheless, their use is limited due to their side effects and costs. On the other hand, butorphanol and nalfurafine are two k-opioid receptor agonists. Butorphanol seems to

be effective in chronic intractable itch associated with inflammatory skin diseases or systemic diseases, while nalfurafine in CKD-associated pruritus ^{244,245}.

1.6.2.4 Neuroleptics

The antipruritic mechanism of gabapentin and pregabalin, structural analogs of the neurotransmitter g-aminobutyric acid (GABA), is unclear, it may be due to the block of nociceptive, and thus pruriceptive, sensations to the brain ². Gabapentine was found useful in neuropathic pruritus (such as brachioradial pruritus and notalgia paresthetica), CKD-associated pruritus, and pruritus associated with cutaneous lymphoma ^{246–248}. However, it is not effective in cholestatic pruritus ²⁴⁹. Also pregabalin has an antipruritic effect on chronic itch conditions, with a risk of withdrawal symptoms if the treatment is stopped abruptly ²⁵⁰.

1.6.2.5 Immunosuppressants

Cyclosporine and azathioprine ameliorate itch in AD probably thanks to their anti-inflammatory effects ^{2,127,251}. The side effects of cyclosporine include hypertension, immunosuppression, elevated creatinine and blood urea nitrogen, and renal toxicity, while with azathioprine there is the risk of dose-dependent myelotoxicity ². In general, the use of immunosuppressants is only recommended for a short period.

1.7. PAPERS AND DISSERTATION OVERVIEW

Study I: Aliotta, G.E., Lo Vecchio, S., Elberling, J., Arendt-Nielsen, L. “Evaluation of itch and pain induced by bovine adrenal medulla (BAM)8-22, a new human model of non-histaminergic itch”. Published to Experimental Dermatology, 2022.

Study II: Aliotta, G. E., Lo Vecchio, S., Elberling, J., Arendt-Nielsen, L. “Effect of antihistaminergic cream doxepin on histaminergic and non-histaminergic itch induced by histamine, BAM8-22, and cowhage”. Submitted, 2022.

Study III: Aliotta, G. E., Lo Vecchio, S., Elberling, J., Arendt-Nielsen, L. “The effect of repetitive topical applications of local anesthetics (EMLA) on experimental pain and itch (histaminergic and non-histaminergic)”. Submitted, 2022.

Characterization of histaminergic and non-histaminergic itch

Characterization of a non-histaminergic itch model based on BAM8-22 (Study I)

Aliotta, G.E., Lo Vecchio, S., Elberling, J., Arendt-Nielsen, L.
"Evaluation of itch and pain induced by bovine adrenal medulla (BAM)8-22, a new human model of non-histaminergic itch".
 Published to Experimental Dermatology, 2022

Comparison between three human surrogate model of histaminergic and non-histaminergic itch, histamine BAM8-22, and cowhage (Study II)

Aliotta, G.E., Lo Vecchio, S., Elberling, J., Arendt-Nielsen, L.
"Effect of antihistaminergic cream doxepin on histaminergic and non-histaminergic itch induced by histamine, BAM8-22, and cowhage". Submitted, 2022.

Modulation of histaminergic and non-histaminergic itch

Antipruritic effects of doxepin on histaminergic and non-histaminergic itch (Study II)

Aliotta, G.E., Lo Vecchio, S., Elberling, J., Arendt-Nielsen, L.
"Effect of antihistaminergic cream doxepin on histaminergic and non-histaminergic itch induced by histamine, BAM8-22, and cowhage". Submitted, 2022.

Effects of repetitive EMLA applications on neurogenic inflammation, mechanical and thermal sensitivities, and histaminergic and non-histaminergic itch (Study III)

Aliotta, G.E., Lo Vecchio, S., Elberling, J., Arendt-Nielsen, L.
"The effect of repetitive topical applications of local anesthetics (EMLA) on experimental pain and itch (histaminergic and non-histaminergic)". Submitted, 2022.

Figure 1: Schematic overview of dissertation studies

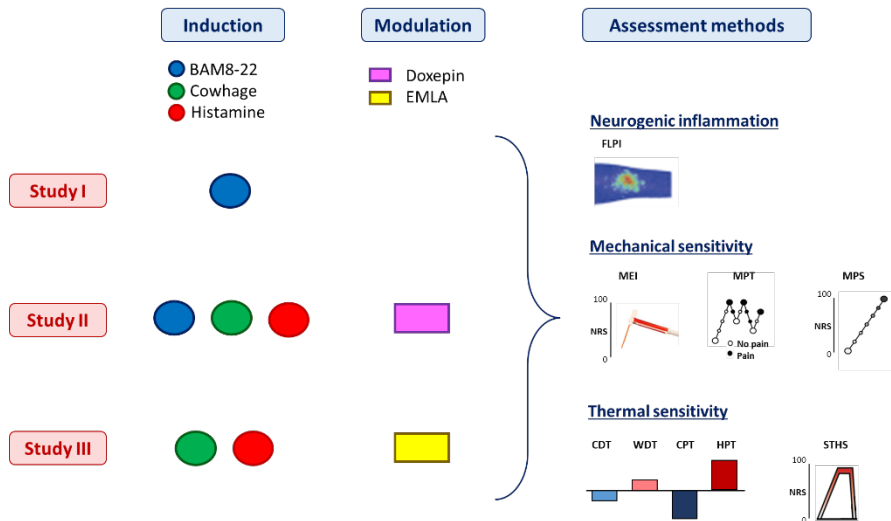


Figure 2: Schematic overview of induction, modulation, and assessment methods used in the three studies.

CHAPTER 2. METHODS

2.1. ITCH AND PAIN ASSESSMENT

The measurement of itch intensity and duration is essential in the study of itch. There are different approaches to assess itch intensity; basically, they involve the usage of severity rating scales, unidimensional assessments of the symptom intensity. The most used severity rating scales are the visual analog scales (VAS), the numeric rating scales (NRS), and the verbal rating scales (VRS). All these scales are useful to also assess pain intensity²⁵².

The visual analog scale was introduced for the measurements of intensities in the 1960s²⁵³, and it is the most used scale for the study of itch²⁵⁴. It consists of a 10 cm line anchored at the two ends. The two ends report a verbal description, which is “no itch” or “no pain” for the left end and “worst imaginable itch” or “worst imaginable pain” for the right end. Patients are instructed to mark on the line, then the investigator will measure the distance between the mark and the left end to settle a score. The graphic orientation of the line depends on the reading tradition of the population involved in the study²⁵⁵. In Western countries, the line is usually horizontal, in a Chinese study the vertical orientation showed less error²⁵⁵. A disadvantage of the VAS is the potential difficult usage without supervision or a proper explanation, particularly for children and older patients²⁵⁶. A variation of the VAS, the color analogue scale (CAS), is used to avoid this problem in children. CAS is a vertical VAS that looks like a thermometer with the bottom small and white corresponding to no symptom, and the top wider and red which means the worse imaginable symptom²⁵⁷.

In the following studies, two electronic visual analogue scales (eVAS) have been used (one for itch and one for pain displayed in the same frame of a tablet). They consist of a 10 cm bar with the outer labels defined as “no itch” or “no pain” and “worst imaginable itch” or “worst imaginable pain”. Moreover, a color gradient (from white to black) was related to the intensity of itch or pain. The subjects were instructed to rate itch and pain intensities continuously for 9 minutes. After the recording, the application automatically transformed the data to values from 0 to 100 collected every 5 seconds. The advantage of this technique is the possibility to measure not only the intensity of itch and pain, but also the duration and the temporal profile after the application of the pruritogens.

The numerical rating scale (NRS) is simple to administrate and score²⁵⁶. It consists of a scale from 0 to 10 where 0 corresponds to “no symptom” and 10 to “worst possible symptom”. The scale could be imaginary, and the patients orally rate the perceived level of symptom (as is done in the following studies) or the scale could be a 10 cm line with tick marks every centimeter and, in this case, patients mark on the 11-point

scale the symptom severity^{252,254}. After a comparison between VAS and NRS, NRS resulted more sensitive in the investigation of treatment effects²⁵⁶. Moreover, it resulted to have better compliance, responsiveness, and applicability for pain rating in the palliative care setting²⁵⁸.

In contrast to VAS and NRS, verbal rating scale (VRS) does not involve numbers to rate the symptom. VRS is a list of adjectives to describe the severity of the symptoms and the most common are “none”, “mild”, “moderate”, and “severe”²⁵⁴. The patients are instructed to choose one definition to describe their perception of the symptoms. Unlike VAS and NRS, only non-parametric tests can be used to analyze the outcome of VRS.

2.1. ASSESSMENT OF SUPERFICIAL BLOOD PERFUSION

After the exposure to chemical irritants, in particular pruritogens, two local cutaneous vasomotor responses, wheals, and neurogenic inflammation can be observed^{118,141,259,260}. The wheal reaction forms around the provoked skin area and it is a circumscribed, pale, swollen dermal edema⁵¹. The wheal is a common consequence of exposure to histamine that acts on capillary receptors with consequent microvascular leakage and acute protein extravasation in the dermis^{141,261–265}. The wheal reaction is non-neuronal, and in fact, it is not affected by anesthesia or ablation of local cutaneous nerves^{263,266}. The wheal reaction is a signal of histaminergic pathway and the capillaries responsiveness to histamine; this is the reason why wheal is used in clinic to assess allergic sensitization to putative allergens^{141,267,268}. Measurement with a ruler of the longest diameter and the orthogonal one is useful to evaluate the wheal reaction^{262,268}.

The neurogenic inflammation or flare is an increase in the superficial blood perfusion (SBP) which occurs as a result of the retrograde signaling from the activation of dermo-epidermal peptidergic nerve fibers^{118,260,269}. It can appear in the area immediately surrounding the application site of an irritant or in the unprovoked surrounding area and, in this case, it is called “secondary” neurogenic inflammation or axon-reflex flare^{99,184,270}. Neurogenic inflammation depends on the peptidergic dermo-epidermal fibers function. It can be almost completely abolished by local infiltration of anesthetics or termini ablation induced by capsaicin, but the extent or severity of the neurogenic flare is not affected by a proximal nerve anesthetization²⁷⁰. Peptidergic C-fibers mostly mediate the flare reaction, in particular CMi-fibers, with their extensive terminal branching and so the possibility to release vasodilatory neuropeptides (such as CGRP and substance P^{264,269,271}) far from the activation point^{117,153,271}. In contrast, PmC-fibers less contribute to the generation of the homotopic flare, and, partially due to the limited extension of their terminal arborizations, they are not involved in the axon-reflex phenomenon^{117,153,271}. For these reasons, a robust

axon-reflex flare is indirect evidence of the CMi-fibers activation, while a weak homotopic flare implicates the PmC-fibers activation.

The application of histamine induces a very robust neurogenic inflammation, and the axon-reflex flare size response was found to be positively correlated with the itch intensity, indicating a strong association between the efferent reaction of the CMi-fibers and the evoked itch perception^{137,156,176,261}. On the other hand, cowhage evokes no or very modest inflammatory response with sporadic micro-wheals and micro-erythematic reactions within the insertion area of the spicules^{156,157,162}.

The neurogenic flare can be visible as an erythematic area around the application site. The border of this reaction is highly irregular, the redness tends to blench centrifugally, and the appearance is different due to the skin tone. For all these reasons, a manual evaluation of the area by drawing the estimated circumference on a transparent sheet is not easy nor accurate. To better analyze both the area and the intensity of the neurogenic inflammation, several perfusion-imaging techniques were developed such as doppler flowmetry, infrared thermography, spectrophotometry, or speckle contrast imaging/full-field laser perfusion imaging (FLPI)^{107,272,273}. In the following studies, FLPI was used to assess neurogenic inflammation. In this technique, a preset laser light pattern illuminates a skin area. The laser light wavelength is around 750 nm and it is just above the visible light wavelength, within the hemoglobin reflectance spectrum²⁷⁴⁻²⁷⁶. The laser light induces a reflection from the investigated skin area that causes a contrast laser pattern or a “speckle pattern”, evaluated in proximate real-time. The speckle pattern shows a lower contrast in relation to an increased cutaneous blood flow²⁷⁵. The good reliability of FLPI is estimated from clinical studies in which blood flow was monitored²⁷⁶⁻²⁷⁸.

2.2. MECHANICAL SENSITIVITY

Mechanical, thermal, and chemical stimulations represent the best options to assess cutaneous dysesthesias in humans for both experimental and clinical research^{94,153}. In contrast to pain dysesthesias, itch dysesthesias have been only poorly investigated. In humans, they can be evaluated in the lesion/provocation site, the primary area, or the region surrounding the lesion/provocation site, the secondary area^{45,49,80,279,280}. To map the dysesthetic area, the mechanical assessment is conducted centripetally towards the lesioned/provoked area in small increments (0.5-2 cm)^{38,45,80}. For alloknesis and allodynia, the participants report when the innocuous stimulus becomes itchy/painful, while for hyperknesis and hyperalgesia, the participants report when the slightly itchy/slightly painful stimulus becomes itchier/more painful^{38,51}. To quantify the dysesthetic intensity another approach is used. In the proximity or within the lesioned/provoked area the stimulus (typically medium-intensity von Frey filaments or pinprick stimulation) is delivered repeatedly using different intensities and the

participants rate the mechanically evoked itch/pain intensity^{61,76,80,156,281}. All these methods have the limitation of relying only on a subjective quantification of the stimulus intensity^{38,43,60,281,282}.

Histamine applications evoke, in non-human primates, allodynia (increased response to stroking) and hyperknesis (increased response to a punctate skin stimulus)^{101,144}. The brush strokes mostly activate A β -fibers and never induce itch under normal conditions, for this reason, a central sensitization phenomenon should occur when itch is provoked⁵¹. Hyperknesis is usually assessed by using pinprick stimulators or filaments^{45,80}, even though the mechanism is unclear, and the afferents are still unknown^{45,283}. The secondary pinprick hyperalgesia is mediated by type-I A δ -fibers through a central mechanism, so it is probably also the mechanism of hyperknesis⁵¹. Moreover, a PmC-fibers contribution can be speculated due to the evidence of the itch 0.5-2 second delay usually reported after a pricking stimulus^{60,174}.

In the studies of the present dissertation, mechanically evoked itch (MEI) was assessed through the use of von Frey filaments (North Coast Medical, Gilroy, CA, USA). The three filaments had 9.8, 13.7, and 19.6 mN of force (size 4.08, 4.16, and 4.31, respectively). The filaments were selected following a study in which it was demonstrated that these are the optimal forces for evoking mild itch at baseline (and thus hyperknesis) without any pain perception and below the known average mechanical threshold for PmC-fibers in humans¹⁵⁶. In the following studies, three stimuli (composed of three pricks in short succession) were conducted with each filament and participants rated the itch intensity on an NRS. The statistical analysis was performed on the total average of the stimuli.

The mechanical pain threshold (MPT) and the mechanical pain sensitivity (MPS) were assessed using a pinprick set (MRC Systems GmbH, Germany) composed of seven needles (diameter of 0.6 mm) with different forces (8, 16, 32, 64, 128, 256, and 512 mN). The MPT was assessed by performing five ascending/descending series of stimuli (rate of each stimulus: 2 s on and 2 s off). The participants reported when the stimulus become painful. The statistical analysis was performed on the geometric means of the five thresholds obtained. On the other side, to assess the MPS, each pinprick was applied in ascending order and the participants rated the pain on an NRS elicited by each stimulus. The same procedure was repeated, and the final mean is the arithmetic mean of the reported pain ratings.

2.3. THERMAL SENSITIVITY

The role of transient receptor potential (TRP) ion channels in itch perception was largely discussed. TRP channels are a heterogeneous group in the family of voltage-gated ion channels. Most of them are organized as six transmembrane segments and

allow the Ca^{2+} to enter in the cell ²⁸⁴. In the field of itch, the TRP channels can act as receptors or amplifying channels. In both cases, the activation and opening of TRPs induce a neuronal membrane depolarization with the consequent start of neuronal signaling that results in itch sensation ²⁸⁴. TRPs can be directly activated by stimuli and then elicit itch. An example is the stimulation of H_2O_2 that directly activate TRPA1 inducing itch, or TRPA1 agonist allyl isothiocyanate which causes also pain ^{285,286}. More often, TRPs in itch transduction act as amplifiers: pruritogens bind and activate receptors expressed in the cell surface and the signaling cascade initiated induces the opening of TRPs. As mentioned above in this dissertation, TRPV1 and TRPA1 seem particularly involved in histaminergic and non-histaminergic itch pathways, respectively.

In the studies of the present dissertation, thermal sensitivity was investigated to evaluate if the application of pruritogens and possible therapies can affect the function of some TRPs. In particular, TRPV1 is activated by heat ($>43^\circ\text{C}$), TRPV3 and TRPV4 by moderate warm ($>30\text{--}39^\circ\text{C}$ and approx. $25\text{--}34^\circ\text{C}$ respectively), while TRPM8 by moderate cool temperature ($<23\text{--}28^\circ\text{C}$) ^{219,287–294}. Moreover, also TRPA1 may be involved in cold sensitivity, even though its function is controversial ^{295–298}.

Cold detection threshold (CDT), warm detection threshold (WDT), cold pain threshold (CPT), heat pain threshold (HPT), and suprathreshold heat sensitivity (STHS) were assessed by using a thermal stimulator Medoc Pathway (Medoc Ltd, Ramat Yishay, Israel). The stimuli were delivered as described in Figure 3.

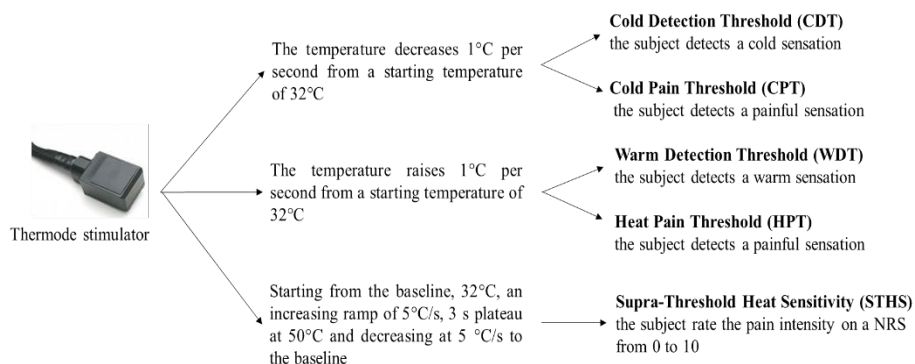


Figure 3: Schematic overview of tests to assess thermal sensitivity.

CHAPTER 3. EVALUATION OF ITCH AND PAIN INDUCED BY BAM8-22

3.1. AIM AND STUDY DESIGN

The aim of the first study was to develop a model of non-histaminergic itch based on the topical application of BAM8-22. For this reason, BAM8-22 was administered by using different delivery methods. In particular, in the first session, different concentrations (BAM8-22 solutions dissolved in distilled water at the concentrations of 0.5, 1, and 2 mg/ml) and vehicle were applied through skin prick test (SPT, performed by applying 120 g of pressure) on 22 healthy subjects. In the second session, the same concentration of BAM8-22 solution (1mg/ml) was applied with different amounts of SPT (1,5, and 25) and using heat-inactivated cowhage spicules.

The present study also aimed to evaluate the effects of the application of BAM8-22. The itch and pain induced were monitored for 9 minutes after the application. Moreover, the neurogenic inflammation, and the mechanical and thermal sensitivities were assessed.

3.1. ITCH AND PAIN INDUCED BY BAM8-22

The results of the present study confirmed BAM8-22 as a good model of non-histaminergic itch in humans, as shown in figure 4. In fact, in both itch peak and area under the curve (AUC) three conditions resulted statistically higher than vehicle: inactivated spicules, 1, and 2 mg/ml (Fig. 4A and B). Moreover, if only the data of the first day are considered, a positive correlation between the BAM8-22 concentration and the itch intensity elicited has been found and this confirms the results of a previous study by Sikand et al., in which BAM8-22 was applied through inactivated spicules soaked in 0.004, 0.04, 0.4, or 4 mg/ml solution and the AUC of itch increased depending on the concentration¹⁶⁸. In both studies, the concentration to saturate the receptors and elicit the highest possible itch intensity was not reached and remains unknown. The baseline BAM8-22 concentration in human skin is not known as well. Up to now, in pathological conditions, such as psoriasis, the expression of proenkephalin A (BAM8-22 precursor) is known to be upregulated in fibroblasts and keratinocytes²⁹⁹. For this reason, proenkephalin A could be considered a contributing factor in the development and maintenance of chronic itch.

Regarding the delivery methods, the SPT application allows BAM8-22 to directly reach the dermo-epidermal junction (approximately 0.5-1.5 mm of depth), while only

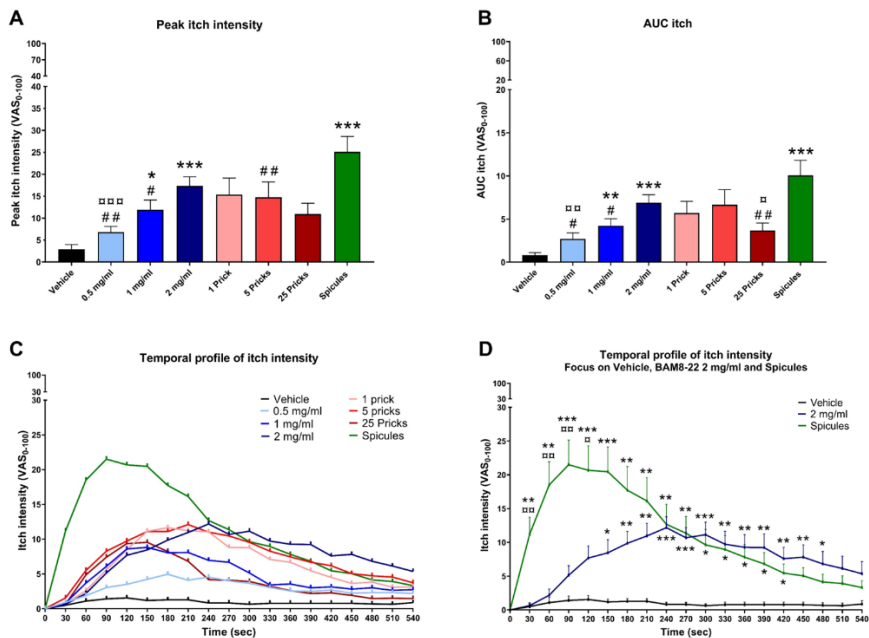


Figure 4: Itch induced by BAM8-22. (A) Peak itch intensity. (B) AUC itch. (C) Temporal profile of itch intensity. (D) Temporal profile of itch intensity focused on BAM8-22 2 mg/ml, inactivated spicules, and vehicle. Values are reported as mean + SEM. Significance indicators: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ vs vehicle; (#) $p < 0.05$, (##) $p < 0.01$ vs BAM8-22 coated spicules; (□) $p < 0.05$, (□□) $p < 0.01$, (□□□) $p < 0.001$ vs BAM8-22 2 mg/ml. Vehicle = black, BAM8-22 0.5 mg/ml = light blue, BAM8-22 1 mg/ml = blue, BAM8-22 2 mg/ml = dark blue, 1 SPT = pink, 5 SPT = red, 25 SPT = dark red, and inactivated spicules = green.

Figure adapted from Aliotta et al. Evaluation of itch and pain induced by bovine adrenal medulla (BAM)8-22, a new human model of non-histaminergic itch. *Exp Dermatol.* 2022

the keratinous layer of the skin (approx. 0.05-0.15 mm) is reached by BAM8-22 applied with the spicules^{157,171,176,272}. Experiments on mice demonstrated that itch is transmitted by two subpopulations of neurons, a peptidergic group expressing MrgprA3 and a non-peptidergic one expressing MrgprD³⁰⁰. They innervate two different layers of the mice skin, in particular, the peptidergic subpopulation innervates the stratum spinosum, while the non-peptidergic subpopulation innervates the more superficial stratum granulosum^{167,300}. The present experiment seems to confirm that itch can be elicited by stimulating both layers of the skin, but not with the same intensity. More precisely, as is shown in the graph of the temporal profile (Fig. 4C and D), the inactivated spicules resulted the best delivery methods to induce

itch, with a peak around 90 seconds after the application. Moreover, the BAM8-22-induced itch rapidly decreased after the peak. Probably, the more superficial fibers present in the skin are more sensitive to BAM8-22 and they induce a more intense itch. Another theory that could explain the more intense itch elicited by the spicules regards the size of the area stimulated. In fact, when spicules are used to induce itch, they activate a larger area than a single SPT. In this way, it is enhanced the possibility to activate a higher number of receptors. In the present study, the spatial summation previously suggested¹⁵⁷, was tested through the multiple SPT. The area provoked by the spicules is comparable to the area provoked by the 25 SPT. The results of the present study on itch showed that the 25 SPT did not provoke intense itch as inactivated spicules. To notice, the 25 pricks induced more, even if not significant after a post hoc analysis, intense pain than all the other conditions (Fig. 5). It seems that the pain sensation masks the itch perception. This effect was proposed in previous studies and explained by the activity of the spinal cord Bhlhb5 interneurons^{301–303}. It was observed that in healthy somatosensory system homotopic pain inhibits itch^{301–303}. The neuronal circuit of this inhibition is well established in rodents: interneurons receive input from primary afferent nociceptors and inhibit spinothalamic pruriceptive transmission⁴⁰.

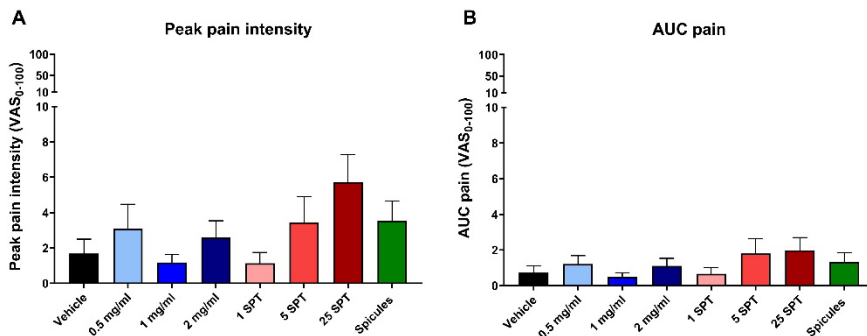


Figure 5: Pain induced by BAM8-22. (A) Peak pain intensity. (B) AUC pain. Values are reported as mean + SEM. Vehicle = black, BAM8-22 0.5 mg/ml = light blue, BAM8-22 1 mg/ml = blue, BAM8-22 2 mg/ml = dark blue, 1 SPT = pink, 5 SPT = red, 25 SPT = dark red, and inactivated spicules = green.

Figure adapted from Aliotta et al. Evaluation of itch and pain induced by bovine adrenal medulla (BAM)8-22, a new human model of non-histaminergic itch. *Exp Dermatol.* 2022

3.2. NEUROGENIC INFLAMMATION

The analysis through the FLPI technique of all the areas reveals an absence of neurogenic flare (Fig. 6). As discussed above in this dissertation, this could be indirect evidence that BAM8-22 induces itch by activating a non-histaminergic pathway without the involvement of CMi-fibers^{51,98,275}. The only differences found concern the 25 SPT and 5 SPT. The superficial blood perfusion of 25 SPT resulted higher than all the other conditions, while 5 SPT-induced SBP was increased only in comparison with vehicle, 0.5, 1 mg/ml, and 1 SPT. The most probable explanation for these changes is a microtrauma to the skin caused by the delivery methods instead of a neurogenic inflammation caused by BAM8-22.

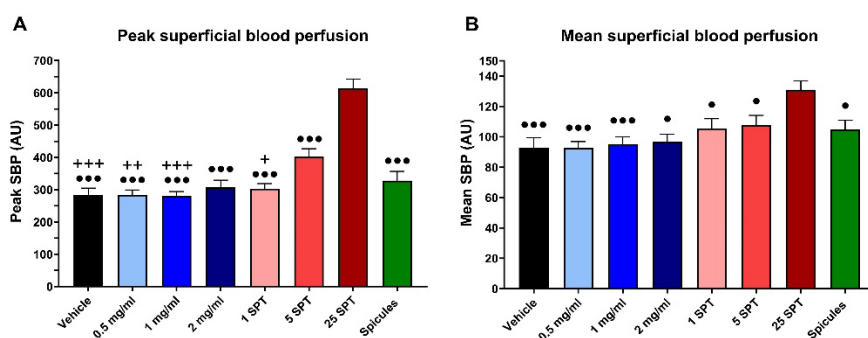


Figure 6: Changes in superficial blood perfusion induced by BAM8-22. (A) Mean SBP. (B) Peak SBP. Values are reported as mean + SEM. Significance indicators: (•) $p < 0.05$, (•••) $p < 0.001$ vs 25 SPT; (+) $p < 0.05$, (++) $p < 0.01$, (+++) $p < 0.001$ vs 5 SPT. Vehicle = black, BAM8-22 0.5 mg/ml = light blue, BAM8-22 1 mg/ml = blue, BAM8-22 2 mg/ml = dark blue, 1 SPT = pink, 5 SPT = red, 25 SPT = dark red, and inactivated spicules = green.

Figure adapted from Aliotta et al. Evaluation of itch and pain induced by bovine adrenal medulla (BAM)8-22, a new human model of non-histaminergic itch. *Exp Dermatol.* 2022

3.3. MECHANICAL AND THERMAL SENSITIVITY

No differences were detected in both mechanical and thermal sensitivities in any conditions compared to vehicle (Fig. 7 and 8). The analysis of the mechanical sensitivity results can detect if the application of BAM8-22 may inhibit or sensitize mechanoreceptors. In the present study, no alterations were found in mechanical sensitivity (Fig. 7). This means that the application of BAM8-22 does not affect A β - and A δ -fibers, stimulated by von Frey filaments and pinprick stimulators respectively

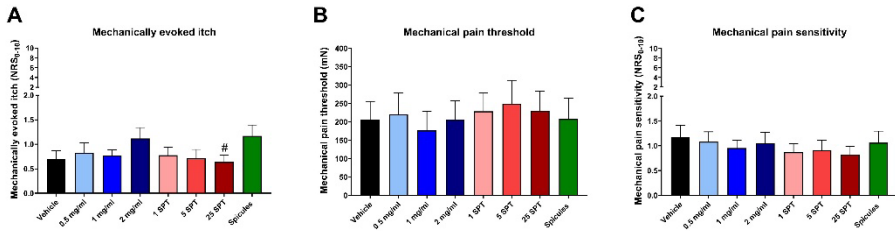


Figure 7: Changes in mechanical sensitivity induced by BAM8-22. (A) Mechanically evoked itch. (B) Mechanical pain threshold. (C) Mechanical pain sensitivity. Values are reported as mean + SEM. Significance indicator: (#) $p < 0.05$ vs BAM8-22 coated spicules. Vehicle = black, BAM8-22 0.5 mg/ml = light blue, BAM8-22 1 mg/ml = blue, BAM8-22 2 mg/ml = dark blue, 1 SPT = pink, 5 SPT = red, 25 SPT = dark red, and inactivated spicules = green.

The thermal sensitivity was not affected as well (Fig. 8). This was not surprising since in previous studies the same result was achieved³⁰⁵. Studies in lesional and/or non-lesional skin of chronic itch patients showed no differences in warm and cold detection^{43,306–308}. In most of them, no changes also in cold pain threshold were found^{43,306,307}, while in all of them the heat pain threshold was not different between lesional and non-lesional skin^{43,306–308}.

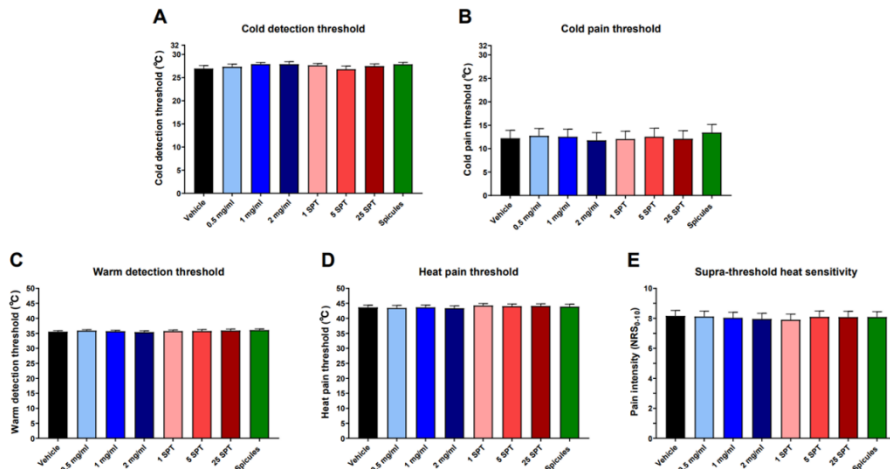


Figure 8: Changes in thermal sensitivity induced by BAM8-22. (A) Cold detection threshold. (B) Cold pain threshold. (C) Warm detection threshold. (D) Heat pain threshold. (E) Supra-threshold heat sensitivity. Values are reported as mean + SEM. Vehicle = black, BAM8-22 0.5 mg/ml = light blue, BAM8-22 1 mg/ml = blue, BAM8-22 2 mg/ml = dark blue, 1 SPT = pink, 5 SPT = red, 25 SPT = dark red, and inactivated spicules = green.

CHAPTER 4. EFFECT OF DOXEPIN ON HISTAMINERGIC AND NON-HISTAMINERGIC ITCH

4.1. AIM AND STUDY DESIGN

The second study aimed to compare the pruritogen properties of histamine, BAM8-22, and cowhage. Moreover, also the neurogenic inflammation and changes in mechanical and thermal sensitivities induced by the three substances were analyzed. To do that, in the first session, histamine, BAM8-22, cowhage, and vehicle were administered in four areas on the forearms of 22 healthy subjects. The itch and pain induced were monitored for 9 minutes with a VAS. At the end of the 9 minutes, neurogenic flare, and mechanical and thermal sensitivities were assessed and compared.

The second aim of the present study was to evaluate the antipruritic effect of 1½ hours-application of doxepin cream on itch and pain induced by histamine, BAM8-22, and cowhage. It was also evaluated if the pretreatment with doxepin could alter the neurogenic inflammation, and the mechanical and thermal sensitivities. In the second session of the experimental procedure, doxepin cream was applied under occlusion for 1½ hours, and after the removal of the cream, the first session was repeated.

4.1. COMPARISON OF ITCH AND PAIN INDUCED BY HISTAMINE, BAM8-22, AND COWHAGE

Histamine, BAM8-22, and cowhage induced itch compared to vehicle (Fig. 9). The analysis of peak itch intensity and AUC did not reveal any differences between the three pruritogens (Fig. 9A and B). A visual inspection of the temporal profile (Fig. 9C) indicates that cowhage induced the higher itch intensity that peaks around 90 seconds after the application, followed by a rapid decline of the itch perception. The temporal profile of BAM8-22 was very similar to cowhage with a peak slightly lower. On the other hand, histamine peaks around 90-150 seconds after the application, and the decline of the itch perception was slower compared to cowhage and BAM8-22. As mentioned above, histamine induces itch by the histaminergic pathway, while BAM8-22 and cowhage by the non-histaminergic itch pathway. The different pathways activated could explain also the differences observed in the temporal profile, very different between histamine and the other two substances. To notice, all three pruritogens elicited moderate to intense itch with high variability in response among

the subjects. The development of these three experimental human surrogate models is very relevant in the study of itch due to the different receptors directly activated by histamine, cowhage, and BAM8-22: H1R, PAR2/4, and MrgprX1 respectively.

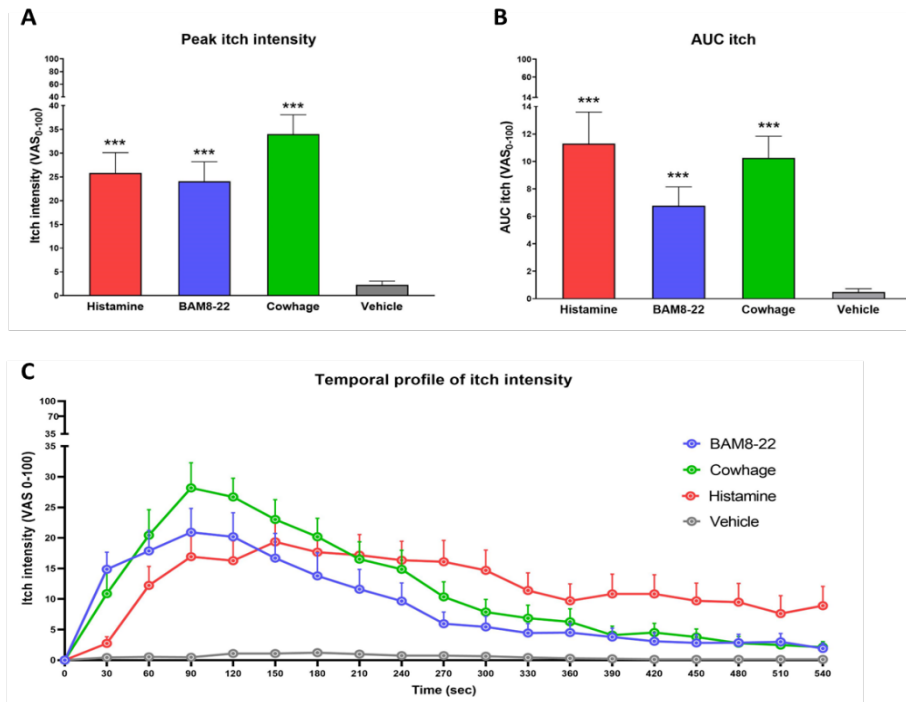


Figure 9: Itch induced by histamine, BAM8-22, and cowhage. (A) Peak itch intensity. (B) AUC itch. (C) Temporal profile of itch intensity. Values are reported as mean + SEM. Significance indicator: (***) $p < 0.001$ vs vehicle. Histamine = red, BAM8-22 = light blue, cowhage = green, vehicle = grey.

Regarding the pain induced by the pruritogens, the statistical analysis reveals an overall difference (Fig. 10), but no specific differences were detected after a post hoc analysis. A visual inspection of the peak and AUC of pain showed that cowhage induced the higher pain intensity, confirming previous studies (see above in this dissertation). The difference with histamine is not surprising, unlike the difference with BAM8-22. In fact, they were supposed to induce a similar pain intensity due to the same non-histaminergic pathway activated. A possible explanation could be a different receptor activation between cowhage and BAM8-22. The results of the present study indicate that the sensations (both itch and pain) elicited by cowhage are more intense than BAM8-22 and this could suggest a stronger activation induced by cowhage.

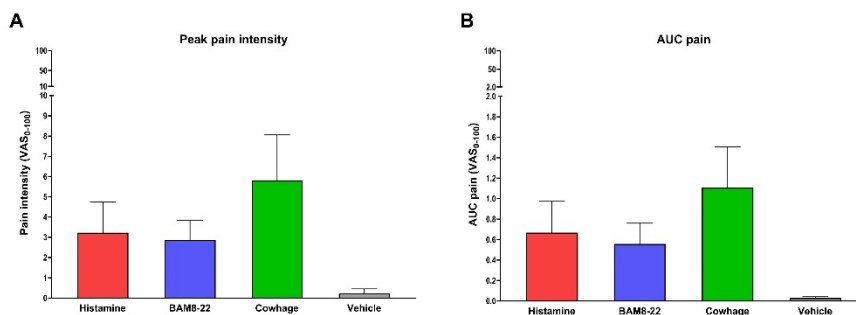


Figure 10: Pain induced by histamine, BAM8-22, and cowhage. (A) Peak pain intensity. (B) AUC pain. Values are reported as mean + SEM. Histamine = red, BAM8-22 = light blue, cowhage = green, vehicle = grey.

4.2. EFFECT OF DOXEPIN ON ITCH AND PAIN

Doxepin, a tricyclic antidepressant (TCA), affects different areas of human body interfering with several pathways and functions. The role as TCA is due to its action at brain level, where doxepin prevents the presynaptic reuptake of serotonin and norepinephrine increasing the synaptic concentration of these neurotransmitters³⁰⁹. Doxepin also acts as an inhibitor of potassium (in cardiomyocytes) and sodium (in cardiomyocytes and at the peripheral level) channels^{310,311}. Moreover, doxepin has strong antipruritic effects due to its ability to block histamine (H1), alpha-1 adrenergic, and muscarinic receptors in the central nervous system³⁰⁹. For this reason, is not surprising that doxepin pretreatment induced a reduction of itch for the three pruritogens (Fig. 11). In particular, histaminergic itch was almost abolished by doxepin as is possible to see in the graphs of peak, AUC, and temporal profile of itch (Fig. 11A, B, and D).

Interestingly, doxepin reduced also the non-histaminergic itch induced by BAM8-22 and cowhage (Fig. 11A, B, E, and F). As is possible to observe in the graphs of temporal profile of itch (Fig. 11C-F), doxepin seems to affect only the intensity and not the duration of non-histaminergic itch. To explain this effect of doxepin on BAM8-22- and cowhage-induced itch, two hypotheses could be formulated. The first one is that non-histaminergic and histaminergic itch pathways have some mechanisms in common. On the other hand, the second hypothesis is that the decrease of non-histaminergic itch could be due to the doxepin block of the sodium channels reducing the action potentials.

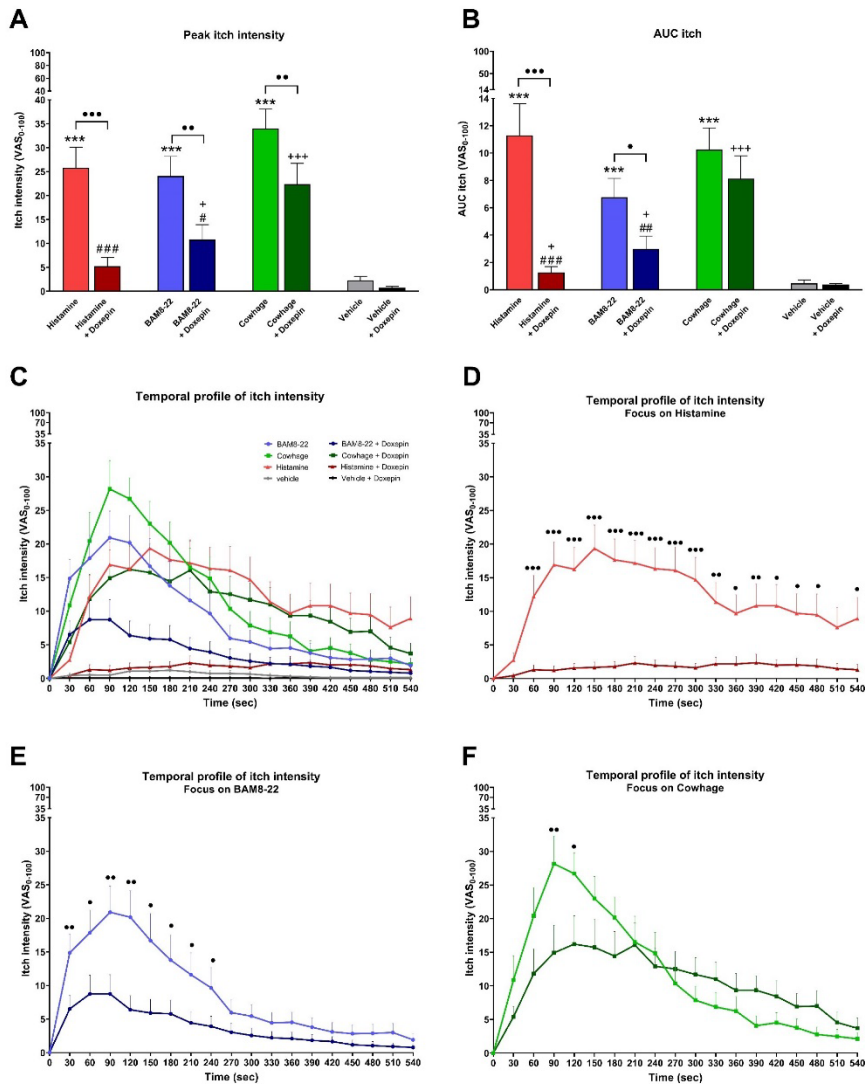


Figure 11: Effect of doxepin on itch induced by histamine, BAM8-22, and cowhage. A) Peak itch intensity; B) AUC itch; C) Temporal profile of itch intensity; D) Temporal profile of itch intensity – Focus on histamine; E) Temporal profile of itch intensity – Focus on BAM8-22; F) Temporal profile of itch intensity – Focus on cowhage. Values are reported as mean + SEM. Significance indicators: (•) $p < 0.05$, (••) $p < 0.01$, (•••) $p < 0.001$ vs doxepin; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ vs vehicle; (+) $p < 0.05$, (+++) $p < 0.001$ vs vehicle+doxepin; (#) $p < 0.05$, (###) $p < 0.01$, (####) $p < 0.001$ vs cowhage+doxepin. Histamine = red, histamine+doxepin = dark red, BAM8-22 = light blue, BAM8-22+doxepin = dark blue, cowhage = green, cowhage+doxepin = dark green, vehicle = grey, vehicle+doxepin = black.

From a visual inspection of the peak and AUC of pain, doxepin seems to reduce pain especially induced by cowhage and histamine (Fig. 12). Also, in this case, this could be due to the block of the sodium channel.

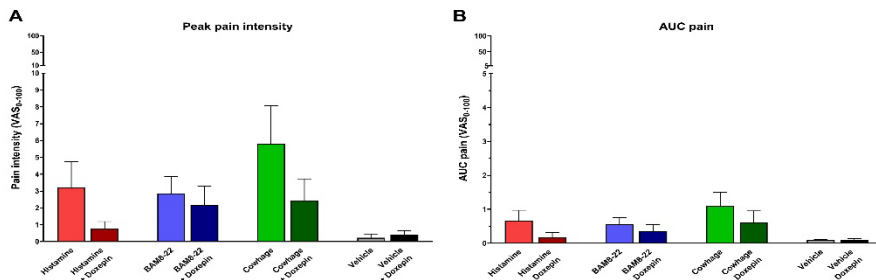


Figure 12: Effect of doxepin on pain induced by histamine, BAM8-22, and cowhage. A) Peak pain intensity; B) AUC pain. Values are reported as mean + SEM. Histamine = red, histamine+doxepin = dark red, BAM8-22 = light blue, BAM8-22+doxepin = dark blue, cowhage = green, cowhage+doxepin = dark green, vehicle = grey, vehicle+doxepin = black.

4.3. NEUROGENIC INFLAMMATION INDUCED BY PRURITOGENS AND THE EFFECT OF DOXEPIN

The analysis of the superficial blood perfusion was used to evaluate the neurogenic inflammation. As mentioned before in Chapter 2, a retrograde signaling from the dermo-epidermal peptidergic nerve fibers causes neurogenic inflammation^{157,260,269}. Moreover, the primary mediators of vasodilatation, CGRP and substance P, were proposed to be involved in the neurogenic flare^{264,269,271}. The previous knowledge of a major contribution of CMi-fibers in flare generation was confirmed by the present study. Histamine induced a largely higher SBP and flare area compared to BAM8-22, cowhage, and vehicle (Fig. 13). The little flare present in BAM8-22 and cowhage areas could be due only to the delivery methods since it is present also in the vehicle area.

Doxepin pretreatment reduced the SBP of all pruritogens, with a predominant effect on histamine (Fig. 13). Moreover, only the flare induced by histamine decreased in size (Fig. 13C). In particular, doxepin has only a minor effect on the peak of SBP induced by histamine, while it has a very bigger effect on the mean SBP and flare area. These two different effects could indicate a slight effect on the homotopic flare and a strong effect on the axon-reflex flare. In addition, previous studies found a positive correlation between itch intensity and axon-reflex flare size mediated by CMi-fibers, and the present results seem to confirm the previous knowledge^{137,156,176,261}.

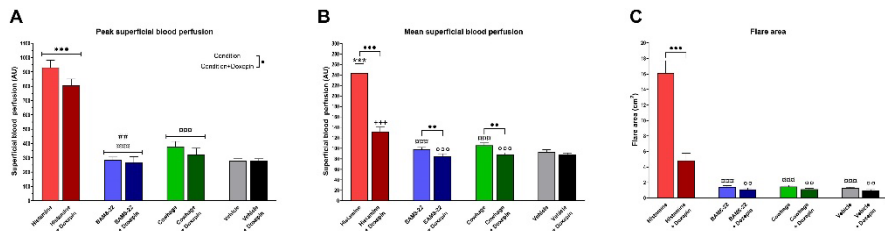


Figure 13: Changes in superficial blood perfusion induced by pruritogens and doxepin. (A) Peak SBP. (B) Mean SBP. (C) Flare area. Values are reported as mean + SEM. Significance indicators: (•) $p < 0.05$, (••) $p < 0.01$, (•••) $p < 0.001$ vs doxepin; (***) $p < 0.001$ vs vehicle; (+++) $p < 0.001$ vs vehicle+doxepin; (#) $p < 0.05$, (##) $p < 0.01$, (###) $p < 0.001$ vs cowhage+doxepin; (□□□) $p < 0.001$ vs histamine; (◊◊) $p < 0.01$, (◊◊◊) $p < 0.001$ vs histamine+doxepin. Histamine = red, histamine+doxepin = dark red, BAM8-22 = light blue, BAM8-22+doxepin = dark blue, cowhage = green, cowhage+doxepin = dark green, vehicle = grey, vehicle+doxepin = black.

4.4. MECHANICAL AND THERMAL SENSITIVITIES

Mechanical sensitivity was assessed through mechanically evoked itch (MEI), mechanical pain threshold (MPS), and mechanical pain sensitivity (MPS) (Fig. 14). The MEI results of the present study indicate that only histamine evoked a higher itch intensity in comparison with vehicle (Fig. 14A). The pretreatment with doxepin reduced MEI in both histamine and BAM8-22 areas, without affecting cowhage and vehicle areas (Fig. 14A). The explanation for this reduction could be that doxepin may decrease peripheral transmission and, in this way, prevent central sensitization, mostly observed in histamine area. Regarding the pain sensitivity, no alterations were found in MPT, while an increased MPS was found after the application of histamine (Fig. 14B and C). Doxepin induced an overall decrease in MPS, probably due to its action on sodium channels on C-, A β -, and A δ -fibers.

The thermal sensitivity was not affected by the application of the three pruritogens, nor by doxepin pretreatment (Fig. 15) indicating that all these substances did not interfere with the TRPs functions. In cold detection threshold (Fig. 15A), a reduction induced by doxepin can be observed, additional studies are needed to verify if this decrease is due to doxepin properties or, more likely, to subjects' habituation to the test.

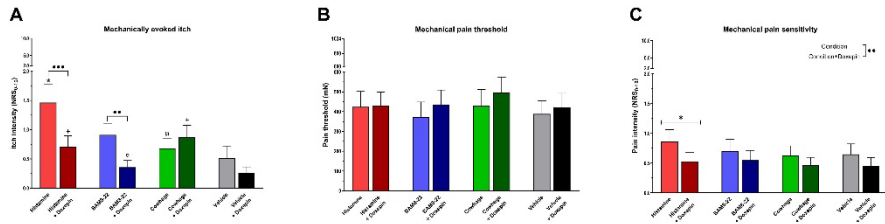


Figure 14: Changes in mechanical sensitivity induced by pruritogens and doxepin. (A) Mechanically evoked itch. (B) Mechanical pain threshold. (C) Mechanical pain sensitivity. Values are reported as mean + SEM. Significance indicators: (**) $p < 0.01$, (***) $p < 0.001$ vs doxepin; (*) $p < 0.05$ vs vehicle; (+) $p < 0.05$ vs vehicle+doxepin; (□) $p < 0.05$ vs histamine; (○) $p < 0.05$ vs histamine+doxepin. Histamine = red, histamine+doxepin = dark red, BAM8-22 = light blue, BAM8-22+doxepin = dark blue, cowhage = green, cowhage+doxepin = dark green, vehicle = grey, vehicle+doxepin = black.

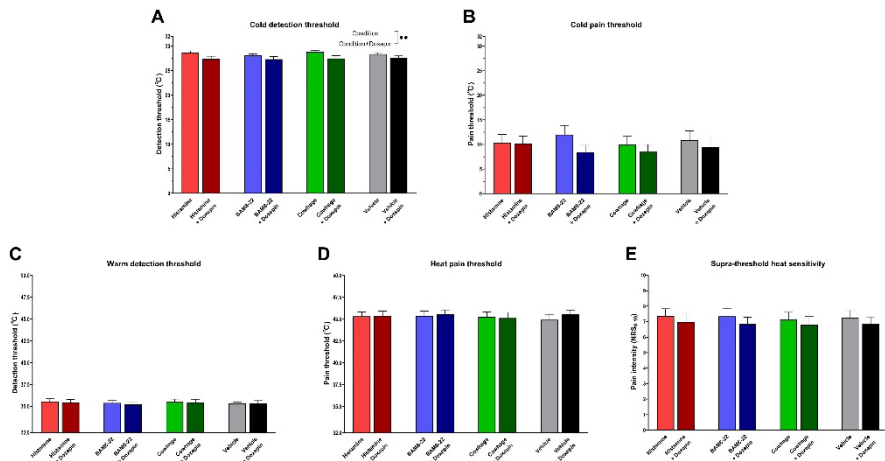


Figure 15: Changes in thermal sensitivity induced by BAM8-22. (A) Cold detection threshold. (B) Cold pain threshold. (C) Warm detection threshold. (D) Heat pain threshold. (E) Supra-threshold heat sensitivity. Values are reported as mean + SEM. Significance indicator: (**) $p < 0.01$ vs doxepin. Histamine = red, histamine+doxepin = dark red, BAM8-22 = light blue, BAM8-22+doxepin = dark blue, cowhage = green, cowhage+doxepin = dark green, vehicle = grey, vehicle+doxepin = black.

CHAPTER 5. EFFECT OF REPETITIVE APPLICATIONS OF EMLA CREAM ON PAIN AND ITCH

5.1. STUDY DESIGN

The third study of the present dissertation aimed to evaluate if repetitive EMLA cream application resulted in a cumulative effect on mechanical and thermal sensitivities. To do that, EMLA was applied to two areas of the forearms of 24 healthy subjects. Two more areas were treated with a placebo cream. EMLA and placebo were applied for three hours under occlusion. At the end of the application time, the creams were removed and SBP, mechanical and thermal sensitivities were assessed. After that, the creams were applied for three more hours followed by the same measurements. This procedure was repeated in the two following days.

The other aim of this study was to evaluate if repetitive EMLA application affected histaminergic and non-histaminergic itch. For this reason, at the end of the third day, histamine and cowhage were applied and itch, pain, and changes in SBP were evaluated.

5.1. CHANGES IN MECHANICAL AND THERMAL SENSITIVITIES AFTER EMLA APPLICATIONS

EMLA is a local anesthetic that blocks sodium channels reducing the transmission of the action potentials^{312,313}. More specifically, when a local anesthetic binds the sodium channels, they pass to an “inactivated state”. From this state it is impossible to directly pass to the “open channel” and for this reason, the opening of Na⁺ channels is reduced with a consequent block of the action potentials³¹⁴. This effect occurs also in A δ - and C-fibers³¹⁴, so it is not surprising that 3-hours of EMLA application induced a reduction in the mechanical sensitivity. In fact, both the mechanically induced itch and pain were reduced in EMLA-treated areas (Fig. 16A-C). To notice, a cumulative effect of EMLA application is not present in this study, the itch and pain experimentally evoked did not further decrease with more EMLA application nor subjects completely stopped to feel mechanical stimuli.

Regarding the thermal sensitivity, similar results were obtained (Fig. 16D-F). A reduction of the thermal sensitivity (corresponding to an increase in warm detection

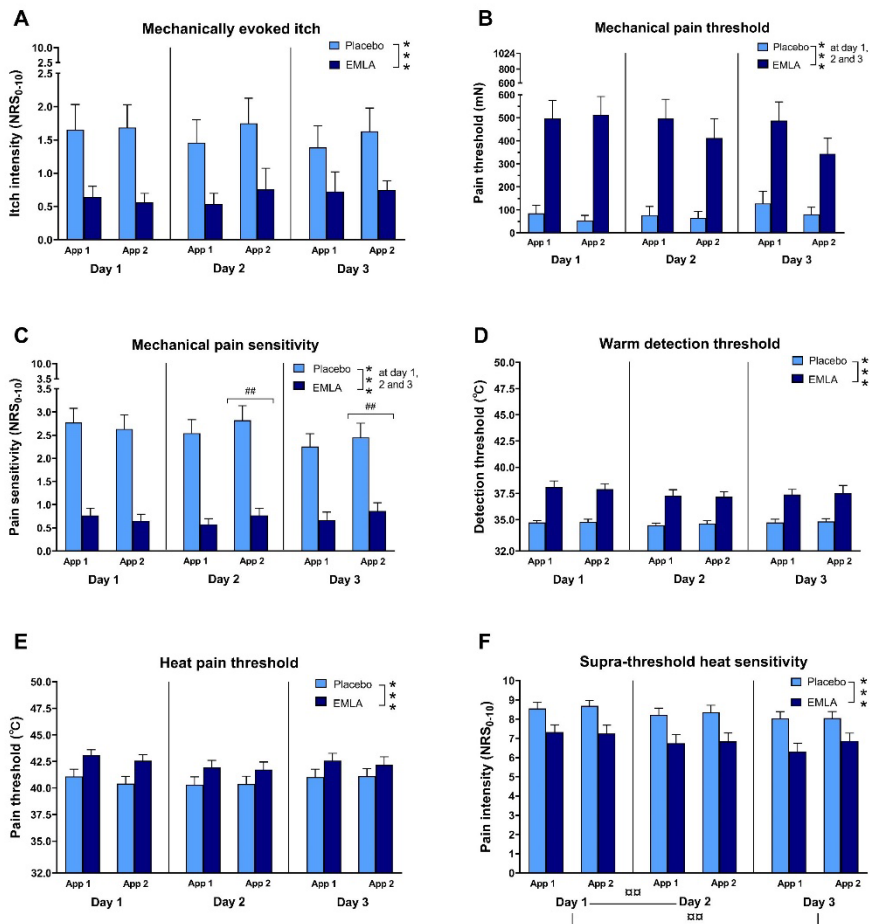


Figure 16: Changes in mechanical and thermal sensitivity after the application of EMLA. A) Mechanically evoked itch; B) Mechanical pain threshold; C) Mechanical pain sensitivity; D) Warm detection threshold; E) Heat pain threshold; F) Supra-threshold heat sensitivity. Values are presented as mean + SEM. Significance indicators: (***) $p < 0.001$ vs placebo; (#) $p < 0.05$, (##) $p < 0.01$ application 1 vs application 2; (□□) $p < 0.01$ difference between days. Placebo = blue and EMLA = dark blue.

threshold and heat pain threshold, and a decrease in supra-threshold heat sensitivity) was present after a single EMLA application. For the thermal sensitivity, an absence of cumulative effect was easier to determine: during the whole procedure (2 x 3-hrs applications x 3 consecutive days) the temperature reached in WDT and HPT remained constant. In STHS there was a reduction among days, but it was probably

due to the habituation to the test since the same reduction was present equally in placebo and EMLA treated areas. The effect of EMLA on thermal sensitivity is probably due to its effect on TRP channels. It is known that lidocaine directly activates the TRPA1 and TRPV1 channels enhancing the calcium influx^{315,316}. A prolonged application may induce desensitization of these channels³¹⁶. The desensitization of TRPV1 could be due to the local anesthetic-induced increase of PLC activity that causes a depletion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂, needed for the TRPV1 activation by lidocaine)³¹⁶.

From the results of mechanical and thermal sensitivities and the absence of a cumulative effect, it is possible to speculate that EMLA application for 3 days did not cause a substantial decrease in fiber density. A reduction of ~40% in fiber density was observed in a previous study in which EMLA was applied for 42 consecutive days³¹⁵. This reduction was observed only in the superficial layers of the epidermis causing only a minimal change in the outcome in the psychophysical tests. It was proposed that a more substantial loss of epidermal and dermal nerve fibers can affect the psychophysical tests^{315,317}.

5.2. EFFECT OF EMLA APPLICATIONS ON ITCH AND PAIN INDUCED BY HISTAMINE AND COWHAGE

In the present study, EMLA pretreatment only induced a reduction of non-histaminergic itch without affecting histaminergic itch (Fig. 17A-C). A visual inspection of the graph of the temporal profile of itch (Fig. 17C) reveals that EMLA reduced the itch intensity of cowhage, and the result of this reduction was that histamine and cowhage had a very similar profile of itch intensity. This effect could be explained by a partial share of mechanisms between histaminergic and non-histaminergic itch pathways. Another possible explanation is the different locations of the PmC- and CMi-fibers in the skin^{138,313,318}. The PmC-fibers are more superficial, while CMi-fibers are located deeper into the vascularized dermis^{51,117}. Cowhage and histamine and their delivery methods reflect the different skin layers to reach. In particular, SPT arrives deeper than spicules into the skin and the different molecular weight (cowhage ~36 kDa vs histamine ~0.11 kDa) allows histamine to diffuse in a less superficial layer than cowhage^{51,174}. It may be possible that the different effect of EMLA on histaminergic and non-histaminergic itch is because the PmC-fibers are more superficial and so more affected by EMLA. A longer application of EMLA may result in a reduction of both histaminergic and non-histaminergic itch due to the decreased fiber density.

Regarding the pain induced by pruritogens (Fig. 17D-F), cowhage induced a higher pain than histamine, confirming previous knowledge (see above in this dissertation).

As expected, the pretreatment with a local anesthetic reduced the pain induced by cowhage, even though it is still present (Fig. 17D-F).

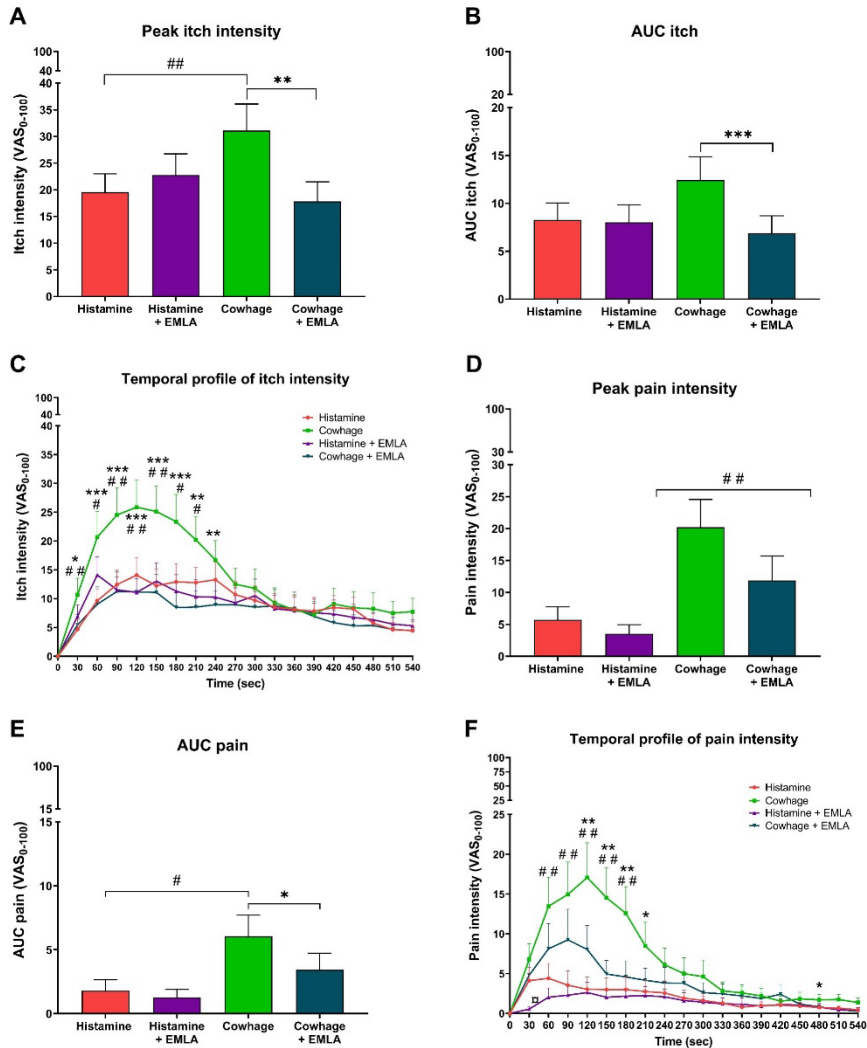


Figure 17: Itch and pain intensities induced by histamine and cowhage after EMLA pretreatment. A) Peak itch intensity. B) AUC itch. C) Temporal profile of itch intensity. D) Peak pain intensity. E) AUC pain. F) Temporal profile of pain intensity. Values are presented as mean + SEM. Significance indicators: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ vs cowhage + EMLA; (□) $p < 0.05$ vs histamine + EMLA; (#) $p < 0.05$, (##) $p < 0.01$ cowhage vs histamine. Histamine = red; histamine+EMLA = violet; cowhage = light green; cowhage+EMLA = dark green.

5.3. NEUROGENIC INFLAMMATION INDUCED BY HISTAMINE AND COWHAGE AFTER EMLA PRETREATMENT

The application of EMLA did not induce any changes in SBP in the present study, compared to placebo. (Fig. 18A, C, and E). In a previous study, it was proposed that a short-time EMLA application induced concentration-dependent vasoconstriction³¹⁹. In the same study, a biphasic effect on SBP was observed after the application of EMLA: a minimal peak of SBP was detected after 1½ hour of application, while erythema and higher superficial blood perfusion were found after 4 hours of application and an increase of 148% in SBP was found after 6 hours of application³¹⁹. Two possible explanations can be formulated for the differences between this and the previous study. The first one is that 3 hours of application is exactly in the middle of the biphasic response and for this reason, no alterations were present in this study. Moreover, between the two applications on the same day, there was a break of approximately 45 minutes (time to run all the mechanical and thermal tests) and possibly the superficial perfusion was normalized during this time avoiding the cumulative effect of the two applications. Another explanation could be that the technique used in this study is more precise and sensitive than the one used in the previous study (specially constructed fiberoptic scanning reflectance spectrophotometer) run in 1989³¹⁹.

To notice, the EMLA pretreatment induced an increase in SBP and flare area size of both histamine and cowhage (Fig. 18B, D, and F). In AD patients, an increase in erythema and redness was observed after 30-60 minutes of EMLA³²⁰. It was proposed that the higher vascular reaction was due to increased absorption of the cream in AD-affected skin³²⁰. As mentioned before, CMi-fibers are involved in the generation of homotopic neuroinflammation and axon-reflex flare, while PmC-fibers are only weakly involved in homotopic neuroinflammation. It could be speculated that the EMLA pretreatment enhance the engagement of C-fibers in neurogenic inflammation and the release of neuropeptides involved in vasodilatation, such as CGRP and substance P.

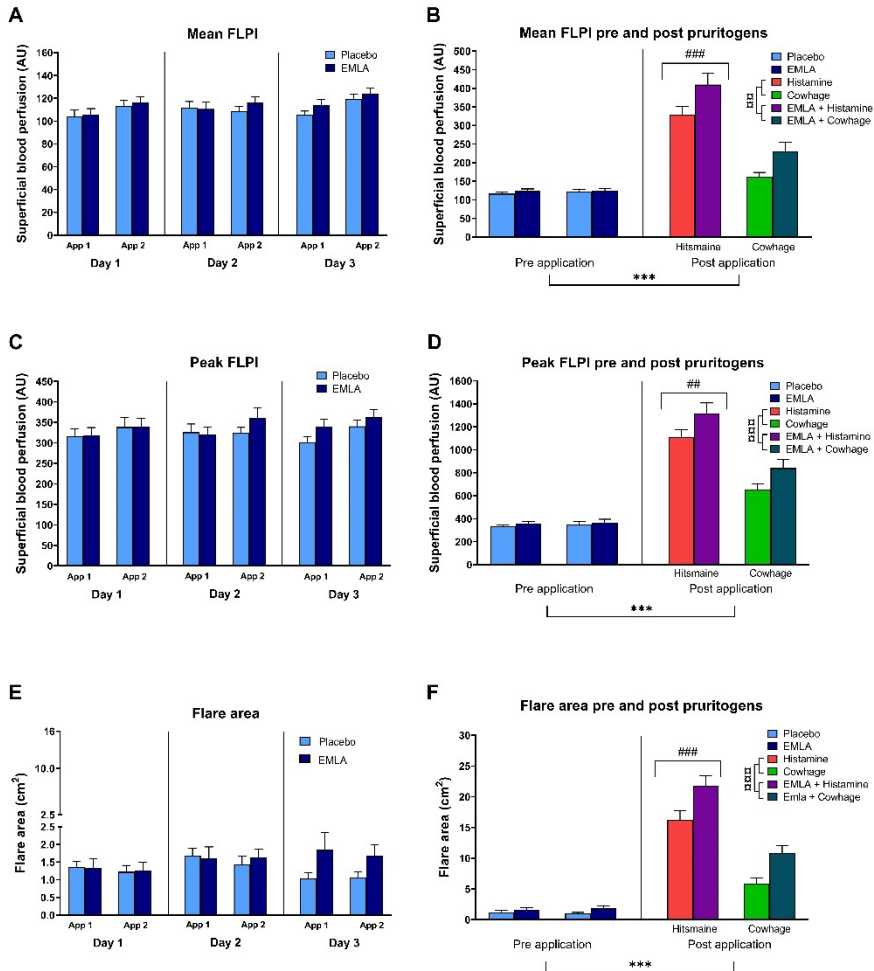


Figure 18: Changes in superficial blood induced by EMLA and pruritogens. (A) Mean superficial blood perfusion; (B) Mean superficial blood perfusion pre and post pruritogens; (C) Peak superficial blood perfusion; (D) Peak superficial blood perfusion pre and post pruritogens; (E) Flare area; (F) Flare area pre and post pruritogens. Values are presented as mean + SEM. Significance indicators: (##) $p < 0.01$, (###) $p < 0.001$ histamine vs cowhage; (□□) $p < 0.01$ vs pruritogens + EMLA; (***) $p < 0.001$ pre vs post application of pruritogens. Placebo = blue; EMLA = dark blue; histamine = red; histamine+EMLA = violet; cowhage = light green; cowhage+EMLA = dark green.

CHAPTER 6. CONCLUSION

In the present PhD project, a novel non-histaminergic itch model based on the application of BAM8-22 was developed. The itch, pain, and the alteration in SBP, mechanical and thermal sensitivities induced by BAM8-22 were compared with the two most used experimental human models to induce itch, histamine and cowhage. Moreover, the effects of two therapies of pruritus currently used in clinic were evaluated in histaminergic (histamine-induced) and non-histaminergic itch (BAM8-22- and cowhage-induced).

In the first study, different concentrations of BAM8-22 and different delivery methods were tested to assess the best one to induce itch. The highest itch intensity was reached by BAM8-22 (concentration of 1 mg/ml) applied through heat-inactivated spicules. The application of BAM8-22 did not induce neurogenic inflammation confirming the activation of the non-histaminergic pathway. Moreover, the itch model based on BAM8-22 did not evoke any kind of pain or mechanical and thermal alterations. In the future, BAM8-22 will be an essential model for the study of itch in humans especially because it seems to evoke pure itch. Moreover, it could be interesting and useful to evaluate the level of BAM8-22 in the skin of chronic itch patients compared to healthy subjects.

In the second study, histamine, BAM8-22, and cowhage were compared. They all induced itch and so can be considered three good acute itch models. Some differences were detected between them; in particular, cowhage application elicited mild pain in addition to itch, while histamine increased the neurogenic inflammation and mechanical sensitivity. These three human models are particularly important for the study of pruritus due to their ability to bind three different families of receptors: H1R located on CMi-fibers, MrgprX1 and PAR2/4 on PmC-fibers. This differentiation is very important in the study of itch features and the development of new therapies. The second part of the experiment focused on the antipruritic effects of doxepin on histaminergic and non-histaminergic itch. It was found that doxepin almost abolished histamine-induced itch, while it only caused a reduction of BAM8-22- and cowhage-induced itch. Moreover, doxepin reduced the increased neurogenic inflammation and mechanical sensitivity induced by histamine. Further studies are necessary to understand if all these effects are due to the doxepin action on the histamine receptors or sodium channels.

In the last study, the effects of repetitive EMLA applications were evaluated in mechanical and thermal sensitivities. EMLA, applied 3 hours twice a day for three consecutive days, induced a reduction of sensitivity without a cumulative effect and without affecting the superficial blood perfusion. At the end of the third day, histamine and cowhage were applied. It was found that EMLA reduced only non-histaminergic itch and pain probably by a selective action on PmC-fibers. Moreover, EMLA

pretreatment enhanced the neurogenic inflammation caused by the pruritogens. Since non-histaminergic itch is the most difficult to treat, these results are particularly relevant for clinical practice.

6.1. FUTURE PERSPECTIVE

The experiments of this PhD project give the opportunity to improve our knowledge in the field of itch, opening new possibilities for the development of therapeutic strategies. In particular, the similarities and differences between two distinct experimental human models of non-histaminergic itch, BAM8-22 and cowhage, should be further investigated to understand if the selective activation of different families of receptors can be found also in pathological itch conditions. Moreover, it could be interesting to evaluate if repetitive applications of pruritogens induce longer and/or more intense itch; in this case, it could be also possible to investigate if some epigenetics modifications occur and compare them with alterations present in chronic itch patients. In addition, the availability of three well-established human itch models targeting three receptor families (Mrgpr, PAR, and histamine receptors) allows for evaluation of new therapeutic targets and strategies, and for the comparison of the effects on different kinds of itch.

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