Validating the peripheral histamine system for therapeutic interventions in pre-clinical *in vivo* models of acute itch and chronic neuropathic pain

By

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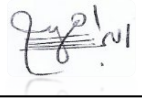
Author's Declaration

This thesis is submitted for the degree of Doctor of Philosophy at institution of translational and clinical research, faculty of medical sciences, Newcastle University. I, **Ibrahim Alrashdi** , hereby confirm that the work presented within this thesis is entirely my own, conducted under the supervision of **Dr.Ilona Obara**, and has not been submitted in any form for another degree at any university or any other tertiary education. Information derived from published and unpublished work of others have been acknowledged in the text and in the list of references given in the bibliography



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LIST OF ABBREVIATIONS

Abbreviation	Description
4EBP1	Eukaryotic translation initiation factor 4E-Binding Protein 1
AC	Adenylate Cyclase enzyme
AD	Alzheimer's Disease
ADHD	Attention Deficit Hyperactivity Disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
ANOVA	Analysis Of Variance
AWERB	Animal Welfare and Ethical Review Body
BBB	Blood Brain Barrier
BNDF	Brain Neurotrophic Derived Factor
Ca^{2+}	Calcium
CaM kinase II	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic Adenosine Monophosphate
CCI	Chronic Constriction Injury
CGRP	Calcitonin Gene-Related Peptide
CNS	Central nervous system
Contra	Contralateral side
COX-2	Cyclooxygenase 2
CPP	Conditioned place preference
CREB	Ca2+/cAMP response element binding protein
CRPS	Complex regional pain syndromes
CYP	Cytochrome P450 enzymes
CYP2D6	Cytochrome P450 2D6
CYP3A4	Cytochrome P450 3A4
DA	Dopamine

DMSO	Dimethyl sulfoxide
DRG	Dorsal Root Ganglion
eIF4E	Eukaryotic Initiation Factor 4E
eIF4G	Eukaryotic Initiation Factor 4 G
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
GABA	g-aminobutyric acid
GAP	GTPase-Activating Protein
GDP	Guanosine Diphosphate
GI	Gastrointestinal
GP	General Practitioner
GPCRs	G protein-coupled receptors
GRK	G-protein receptor kinase
GTP	Guanosine triphosphate
H	Hydrogen
H_1R	Histamine H1 receptor
H_2R	Histamine H2 receptor
H_3R	Histamine H3 receptor
H_4R	Histamine H4 receptor
HIV	Human immunodeficiency virus
IASP	International Association for the Study of Pain
ICSI	Intracytoplasmic sperm injection
i.d.	Intradermal
IL	Interleukin
i.p.	Intraperitoneal
i.pl.	Intraplanter
Ipsi	Ipsilateral side
iGluRs	Ionotropic receptors
JNK	JNK - c-Jun N-terminal kinase
LKB1	LKB1 - Liver kinase B1
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MAPK	Mitogen-activated protein kinase
mGluRs	Metabotropic ligand sensitive receptors
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex-1
mTORC2	mTOR complex-2

MS	Multiple Sclerosis
NAc	Nucleus accumbens
NeP	Neuropathic Pain
NeuPSIG	Neuropathic Pain Special Interest Group
NICE	National Institute for Health and Care Excellence
NIDDM	Non-insulin-dependent diabetes mellitus
NMDA	N-methyl-D-aspartate
NMDARs	NMDA receptors
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NRSF	neuron-restrictive silencer factor
NSAIDs	Nonsteroidal anti-inflammatory drugs
OHSS	Ovarian hyperstimulation syndrome
OIH	Opioid-induced hyperalgesia
ORL1	Opioid receptor like-1
p70S6K	70 kDa ribosomal protein S6 kinase 1
PCOS	Polycystic ovary syndrome
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3 kinase
PIKK	Phosphoinositide 3-kinase (PI3K)-related kinase
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
Rheb	Ras homolog enriched in brain
S6RP	S6 Ribosomal Protein
s.c.	Subcutaneous
SNI	Spared nerve injury
SNL	Spinal nerve ligation
SNPs	Single nucleotide polymorphisms
SP	Substance P
t1/2	Half-life
TNFa	Tumor necrosis factor alpha
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
TrkA	Tyrosine kinase A
TSC	Tuberous sclerosis
UGT	Uridine diphosphoglucuronosyl transferase
UGT2B7	UDP-Glucuronosyltransferase-2B7
ULK1	Protein kinase that initiates autophagy

VLDL	Very low-density lipoprotein
VTA	Ventral tegmental area
UV	Ultraviolet
WB	Western blot
WHO	World Health Organisation
WT	Wild type

RELEVANT ABSTRACTS AND PUBLICATIONS

Journal publications:

1. Obara I, Telezhkin V, **Alrashdi I** and Chazot PL.(2019) Revisiting the histamine system for neuropathic pain relief. British Journal of Pharmacology.
2. **Alrashdi, I.**, Alsubaiyel, A., Chan, M., Battell, E. E., Ennaceur, A., Nunn, M. A., Weston-Davies, W., Chazot, P. and Obara, I. (2022) 'Votucalis, a Novel Centrally Sparing Histamine-Binding Protein, Attenuates Histaminergic Itch and Neuropathic Pain in Mice', Frontiers in pharmacology, 13.

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1. **Alrashdi I**, Alsubayiel A, Chazot P, Nunn M, Weston-Davies W, Obara I. (2018) Novel histamine-binding protein rEV131 attenuates neuropathic pain in mice. British Pharmacological Society annual meeting, Pharmacology 2018, London, UK. **(Poster Session)**

2. Battell E, Alsubaiyel A, **Alrashdi I**, Rosa AC, Chazot PL, Pini A, Liu WLS, Nunn M, Weston-Davies W, Obara I. (2018) Histamine system and neuropathic pain relief: novel strategies. World Histamine Symposium (WHS2018), a satellite symposium of WCP2018, 2nd Joint Symposium of European and Japanese Histamine Research Societies, July 2018, Kobe, Japan.

3. Obara I, Alsubaiyel A, **Alrashdi I**, Battell E, Nunn M, Weston-Davies W, Chazot PL. (2018) Revisting histamine system for neuropathic pain relief: novel natural product strategy. European Histamine Research Society, 47th Annual Meeting, May/June 2018, Dublin, Ireland.

4. Young N, **Alrashdi I**, Battell E, Chazot P, Obara I (2019)The effect of the CNS-sparing histamine H3receptor antagonist; ZPL-8680871 in the treatment of Neuropathic Pain.

5. **Alrashdi I**, A. Alsubaiyel A, Nunn M, W. Weston-Davies W, Chazot PL, Obara I. (2019) Local administration of histamine binding protein EV131 attenuates neuropathic pain and itch in mice: comparison to systemic effects. European Histamine Research Society meeting, EHRS 2019, Krakow, Poland. **(Oral Presentation Session)**

6. **Alrashdi I**, Chazot P, Nunn M, Weston-Davies W, Obara I. (2019) Novel histamine-binding protein rEV131 attenuates itch in mice: comparison of local and systemic effect. British Pharmacological Society annual meeting, Pharmacology 2019, London, UK. **(Poster Session)**

7. Young, N., Pham, L., **Alrashdi, I.**, Obara, I., Telezhkin, V. and Chazot, P., (2020) Hyperglycaemia elicits up-regulation of the histamine H-4 receptor in human retinal Muller cells in vitro. British journal of pharmacology (Vol. 177, No. 11, pp. 2525-2526). 111 RIVER ST, HOBOKEN 07030-5774, NJ USA: WILEY.**(Abstracts)**

8. **Alrashdi I**, Chazot P, Nunn M, Weston-Davies W, Obara I. (2020) Histaminergic Itch And The Role Of Histamine H1 And H4 Receptors In Mice. European Histamine Research Society meeting, EHRS 2020, Krakow, Poland. **(Online Oral Presentation Session)**

9. **Alrashdi I**, Chazot P, Nunn M, Weston-Davies W, Obara I. (2020) rEV131, neuropathic pain, histaminergic itch and the role of histamine H1 and H4 receptors. IASP 2020 World Congress on Pain, Amsterdam,Netherlands. **(Abstract**

IMPORTANT DEFINITION AND TERMINOLOGY ACCORDING TO INTERNATIONAL ASSOCIATION FOR THE STUDY OF PAIN (IASP)

Terms	Definitions
➢ **Allodynia**	Pain due to a stimulus that does not normally provoke pain.
➢ **Analgesia**	Absence of pain in response to stimulation which would normally be painful.
➢ **Central neuropathic pain**	Pain caused by a lesion or disease of the central somatosensory nervous system.
➢ **Central sensitization**	Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input.
➢ **Dysesthesia**	An unpleasant abnormal sensation, whether spontaneous or evoked.
➢ **Hyperalgesia**	Increased pain from a stimulus that normally provokes pain.
➢ **Hyperesthesia**	Increased sensitivity to stimulation, excluding the special senses.
➢ **Hypoalgesia**	Diminished pain in response to a normally painful stimulus.
➢ **Hypoesthesia**	Decreased sensitivity to stimulation, excluding the special senses.
➢ **Neuralgia**	Pain in the distribution of a nerve or nerves.
➢ **Neuritis**	Inflammation of a nerve or nerves.
➢ **Neuropathic pain**	pain that arises as a direct consequence of a lesion or diseases affecting the somatosensory system.
➢ **Nociception**	The neural process of encoding noxious stimuli.
➢ **Nociceptive pain**	Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors.
➢ **Nociceptive stimulus**	An actually or potentially tissue-damaging event transduced and encoded by nociceptors
➢ **Nociceptor**	A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli.
➢ **Noxious stimulus**	A stimulus that is damaging or threatens damage to normal tissues.
➢ **Pain**	An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage
➢ **Pain threshold**	The minimum intensity of a stimulus that is perceived as painful.
➢ **Paresthesia**	An abnormal sensation, whether spontaneous or evoked.
➢ **Peripheral neuropathic pain**	Pain caused by a lesion or disease of the peripheral somatosensory nervous system.
➢ **Peripheral sensitization**	Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields.

ABSTRACTS

The histaminergic system is an interesting target for the development of new anti-pruritic and analgesic medications as all four histamine receptors (H_1R - H_4R) are expressed in regions concerned with itch and pain transmission. Recent advances in itch and pain research have elucidated the potential role of histamine and its receptors in the initiation and maintenance of both conditions. This fast-emerging knowledge about the role of histamine system in a variety of physiological and pathological processes led to the hypothesis that blocking histamine action may have beneficial therapeutic approaches in wide range of pathological conditions, including acute itch (pruritis) and chronic neuropathic pain.

Both neuropathic pain resulting from nerve damage as well as itch where histamine has been a well-known mediator have limited therapeutic strategies due to incomplete understanding of the mechanisms underlying these major clinical problems. Both conditions seem to have a significant negative impact on health-related quality of life and daily functioning, including physical, emotional and social well-being. Pharmacological management remains the most common therapeutic option for both itch and chronic neuropathic pain, but therapeutic outcomes are still poorly achieved. Moreover, systemic therapy is commonly associated with side effects that can also lead to medication safety issues and discontinuation of treatment. Therefore, more effective, and safer treatments for itch and chronic pain are urgently needed. Indeed, local peripheral administration of medications can be a viable alternative to systemic delivery with a lower rate of systemic side effects, reduced interactions with other systematically acting substances, and allow direct targeting of the affected area/s. In line with this therapeutic strategy, peripheral targeting of the histamine system has provided interesting tools for further investigation of the role of histamine and its receptors in itch and chronic pain. Therefore, here I hypothesized that scavenging of peripherally released endogenous histamine or selective blocking of specific type of histamine receptor result in both

anti-itch and anti-nociceptive effects. To test this hypothesis, adult male mice were subjected to histaminergic itch, as well as peripheral nerve injury that resulted in neuropathic pain. I investigated the role of peripheral histamine receptors (HRs) and evaluated the anti-pruritic and anti-nociceptive efficacy of two novel ligands targeting the peripheral histamine system. Specifically, I worked with Votucalis (rEV-131) that is a novel centrally sparing highly affinity histamine scavenger protein as well as PF-0868087 that is a selective and peripherally-acting / centrally-sparing H_3R antagonist. The uniqueness of these novel ligands lies in their chemical structure that restricts penetration to the central nervous system. Thus, these drugs have been ideal candidates to test our hypothesis and to validate peripheral HRs as a potential target for therapeutic intervention in both acute itch and neuropathic pain. Using behavioural *in vivo* models of acute itch and neuropathic pain, I showed for the first time that both Votucalis and PF-0868087 had a significant effect on attenuating acute itch induced in mice by injection of compound 48/80 (is N-methyl-p-methoxyphenethylamine and formaldehyde used to induce mast cell degranulation and histamine release), and peripheral neuropathy induced by chronic constriction injury (CCI) of the sciatic nerve, respectively. Based on the mechanisms of nerve injury-induced hypersensitivity it is possible that PF-0868087 and Votucalis targeted peripheral histamine receptors expressed on subpopulation of A-delta. In addition, I investigated the role of peripheral histamine receptors in acute itch using photo-switchable ligands to control pharmacological activity using a specific wavelength of light. For the first time, I used VUF-16129 a photo-switchable compound which can be reversibly switched via light between two isomers (*trans-cis*) corresponding to high and low pharmacological activity showing the importance of H_4R in the regulation of histaminergic itch.

Taken together, our findings further emphasize the key role for histamine system in the regulation of itch and chronic pain. Importantly, I provide the first evidence that peripheral targeting of the histaminergic system either by scavenging of histamine or blocking the specific type of receptors support the importance of the histamine system in the modulation of peripheral itch and neuropathies and represent a novel therapeutic strategy in the treatment of both conditions

1. INTRODUCTION

1.1 Acute Itch

1.1.1 Definitions and background

Itch (also known as pruritus) is one of the most common symptoms of skin and allergic diseases (Song et al., 2018; Weisshaar and Dalgard, 2009). It is also an unwanted symptom of many systemic disorders such as thyroid disease, renal and hepatic failure, and leukemia (Greaves, 2005). More than 350 years ago, the German medical doctor Samuel Hafenreffer defined itch as an *"unpleasant sensation that elicits the desire or reflex to scratch"* (Ikoma et al., 2006). In fact, this definition is still in use today, and it may be linked to an incomplete understanding of pathophysiology and mechanisms of itch (Cowan and Yosipovitch, 2015; Liu and Ji, 2013). According to several studies, itch was considered as a low-intensity type of pain as both itch and pain sensations are believed to share several common characteristics and mechanisms (Liu and Ji, 2013; Ständer and Schmelz, 2006a). They are similar in transmission pathways from the peripheral nervous system (PNS) to the central nervous system (CNS) involving similar mediators, modulators and receptors (Ikoma et al., 2011; Ikoma et al., 2003; Liu and Ji, 2013). Itch is considered as a normal body response that serves as a warning signal for harmful external agents and internal diseases (Ross, 2011; Liu and Ji, 2013). Itch is shared among several subtypes of animal species such as monkeys, rabbits, guinea pigs, rats and mice (Cowan et al., 1985; Gmerek and Cowan, 1983; Katz, 1980).

Itch can be classified into two types as acute itch (lasting for less than six weeks) and chronic itch (lasting for six weeks or more) based on the chronicity of the symptoms (Ständer et al., 2007). Acute itch is a daily experience commonly controlled by scratching in and around the affected area (Davidson and Giesler, 2010). Also, it can get provoked by an injury or inflammation of the skin due to contact with itch-causing agents or allergens, which lead to activation of pruriceptive neurons at the site of the local tissue area (Ikoma et al., 2006). Whereas chronic itch occurs with a wide range of pathological

skin conditions such as atopic dermatitis (AD), xerosis, psoriasis as well as some systemic disorders such as liver and kidney diseases or metabolic disorders like hyperthyroidism, diabetes mellitus (Bieber and Novak, 2009; Ständer et al., 2007; Potenzieri and Undem, 2012; Yamaoka et al., 2010) or due to neuropathic and psychogenic causes (Yosipovitch and Samuel, 2008; Weisshaar and Dalgard, 2009).

There are limited reports regarding the frequency of occurrence of acute itch in the general population. However, more recent studies suggest that the prevalence of acute itch in general population is about 8.4% (Szepietowski and Weisshaar, 2016) while chronic type of itch is likely to lie between 8% and 38% of global population (Matterne et al., 2013; Misery and Ständer, 2010). According to several prevalence studies that detect women, the elderly, and the people from lower socio-economic groups are the most commonly affected patients (Matterne et al., 2013; Misery and Ständer, 2010).

The underlying causes of acute itch differs markedly from the underlying causes of chronic type of itch (Lerner, 2018). The most common causes of acute itch are: allergic reactions to food or medications, insect bites, and infections (Schaefer, 2017;Lerner, 2018). Chronic itch are likely to be complex, multifactorial and subjective, many internal and external factors are involved in chronic type of itch (Song et al., 2018). The intrinsic factors include chronic infection, hormonal changes, metabolic disturbances, hereditary and genetic factors (Jafferany and Davari, 2019), whereas the extrinsic ones are more complicated, modifiable, and changeable, consisting of inhaled substances, food, sunlight, chemical materials, nicotine, animal hair and fur (Lyell, 1972; Song et al., 2018). These reasons make the treatment of this condition quite difficult (Meyer, Paul and Misery, 2010; Leslie, 2013).

There is a significant absence of knowledge about the pathophysiology of itch because of its complexity in exhibiting the symptoms, numerous aetiologies and lack of strong evidence for the clinical outcome measures (Weisshaar et al., 2012). Recently, a vast amount of research has been done to identify the underlying mechanism of this sensation (Rinaldi, 2019). However, till now our knowledge regarding the underlying mechanism of itch in general, is still not clear and far from complete. Therefore, a comprehensive understanding of the intracellular pathways and the mechanisms that

are involved in the transmission of itch is highly important. Generally, the transmission of itch can be divided into two main neuronal pathways; histaminergic (caused mainly by histamine) and non-histaminergic (caused by several pruritogens such as tryptase and protease) pathways (Yosipovitch et al., 2018 and Kahremany et al., 2020). Acute itch is mediated *via* both pathways (Yosipovitch et al., 2018; Rinaldi, 2019; Kahremany et al., 2020). In contrast, chronic forms of the itch are mostly mediated *via* the non-histaminergic pathway (Yosipovitch et al., 2018; Kahremany et al., 2020). Part of this thesis will focus on the acute itch. More specifically, in this study, we hope to shed light on the role of hisataminergic system in acute type of itch.

Itch, particularly the chronic type, has an undesirable impact on quality of life including physical, mental, and emotional (Verhoeven et al., 2007; Van Os-Medendorp et al., 2008; Whang et al., 2020). Indeed, patients suffering from itch seem to have an extreme psychosocial morbidity and dysfunction (Van Os-Medendorp et al., 2008). Commonly related psychosocial dysfunction include mental distress, agitation, anxiety and depression (Lsheehan-Dare, Henderson and A. Cotterill, 1990; Tey, Wallengren and Yosipovitch, 2013). In addition, many studies found that itch can affect the daily performances and activity of the sufferer, which include concentration, sleep and ability to attend school and work regularly (Tey, Wallengren and Yosipovitch, 2013; Chinniah and Gupta, 2014).

Until today the management of itch is one of the significant challenges for physicians and researchers worldwide. There is a limitation in the efficacy of traditional treatment methods and the advanced treatments methods are yet to be developed (Summey and Yosipovitch, 2005). Generally, treatment of itch is based on its aetiologies and the primary cause of the illness (Rinaldi, 2019). Since the multifactorial nature of the itch symptoms and its wide range of possible aetiologies, a diagnostic workup, and therapeutic approach are of high complexity (Ständer et al., 2015). The treatment of acute itch is usually uncomplicated and direct, however the management of chronic itch can be significantly more complicated and difficult (Leslie, 2013). Current treatment regimens indicate several peripheral or systemic therapies. Generally, peripheral adminstration of the itch medication such as corticosteroids, immunomodulators, capsaicin, and antihistamines are frequently used as first-line therapy due to their lower

risk of adverse effects compared to systemic treatments (Hong et al., 2011). In addition, one of the novel anti-pruritic strategies is the selective targeting of peripheral itch mediators such as histamine, serotonin, bradykinin, and prostaglandin (Misery and Ständer, 2010; Yosipovitch et al., 2019).

Consequently, there is a considerable need to explore novel pharmacotherapies for the successful treatment of itch and this requires a deeper knowledge on the mechanisms related to itch as well as more appropriate tools for assessing acute and chronic itch sufferers. Thus, one novel therapeutic option could be by exploreing selective targeting of the local histaminergic system since it has been suggested that histamine released in the peripheral system could potentially interact with mechanisms that underly the development and maintenance of itch. Therefore, this signifies the importance of a viable beneficial target for the management of this condition and will be explored in the present research.

1.1.2 Clinical classification of itch

The classification of itch remains a topic of controversy and till now there is no worldwide accepted clinical categorization of this condition (Bernhard, 2005; Ikoma et al., 2006). Classification of itch is necessary for accurate diagnostics and effective treatment. Therefore many classifications have been created based on several factors such as time course (chronological approach), mechanism, intensity and location.

Based on the chronological classification, itch can be classified into two main types; this-includes acute and chronic itch. Acute itch is a type of itch lasting up to 6 weeks, and itch lasting 6 weeks or more is termed as chronic itch (Ständer et al., 2007). The mechanistic classification of itch is based on the underlying mechanism that mediates itch *via* PNS or CNS. Itch can be further classified based on the location and distribution over the body into localized or generalized itch (Misery et al., 2018; Reamy et al., 2011).

However, these previous classifications have several limitations to be utilized in clinical application. Example for the limitations of the privious classification systems is that this classification does not include the type of itch of unknown origin (Ständer et al., 2007; Reamy et al., 2011). Also many itch conditions, such as atopic dermatitis (AD) and cholestatic pruritus, fit in more than one category (Ständer et al., 2007). Therefore, in

2007, based on the clinical manifestation the International Forum for the Study of Itch (IFSI) classified itch into five categories (Ständer et al., 2007).

These are: skin-derived pruritus (due to stimulation of pruriceptive at their sensory nerve endings such as urticaria, scabies and insect bite dermatitis), systemic pruritus (occuring due to systemic diseases such as cholestatic itch), neuropathic pruritus (due to diseased or lesioned cause alterations in the afferent pathway of sensory nerve fibers, such as multiple sclerosis), psychogenic pruritus (occuring due to psychologic abnormalities such as itch in a patient with parasitic phobia) and mixed pruritus (occuring due to multi factors and more than two mechanism such as uraemia) (Brennan, 2016; Ständer et al., 2007; Song et al., 2018). Figure 1.1. shows a summary of the common classification system of itch along with common examples.

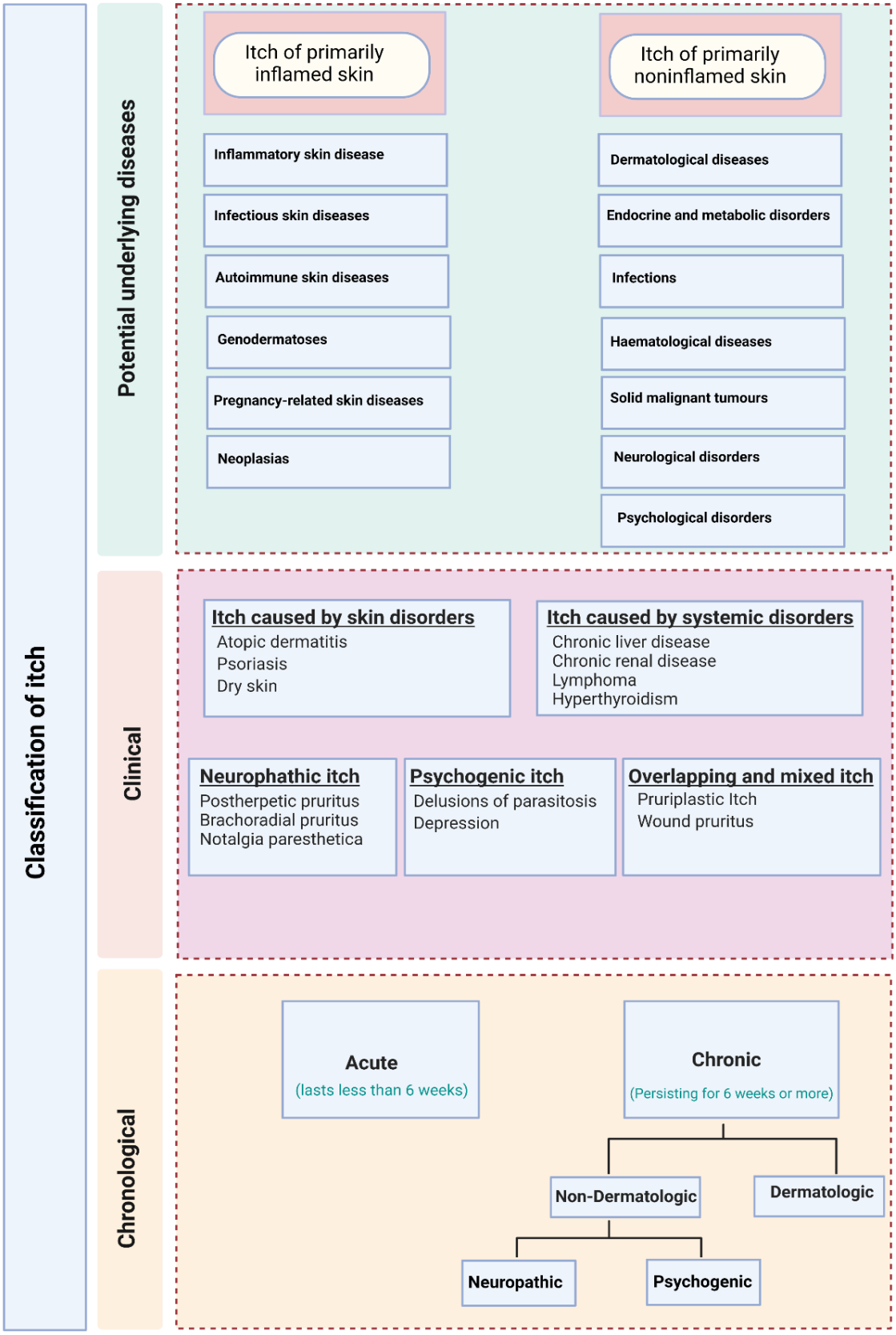


Figure 1.1 A summary of common classification system for itch (adapted from Metz et al., 2011; Ikoma et al., 2006 and Song et al., 2018)

1.1.3 Epidemiology of itch

Itch is one of the most common subjective symptoms in the dermatology field, and it can appear with or without recognizable skin changes (Tarikci et al., 2015). Itch is the frequent presenting complaints and diagnosis of skin problems in primary care centers (Tivoli and Rubenstein, 2009). However, few epidemiological studies have been performed on the prevalence of this condition (Whang et al., 2020). Several clinical studies indicate that patients with acute itch commonly do not consult a physician, while patients with chronic itch more frequently do (Weisshaar and Dalgard, 2009; Whang et al., 2020).

Itch may be experienced due to a wide range of systemic diseases like renal, hepatic, blood, neurological, and psychiatric diseases, but also may occur due to some type of medications taken or may be caused by unknown factors (Weisshaar, 2016). This diversity may explain why itch's epidemiological research is very challenging and has long been ignored (Whang et al., 2020). A new European epidemiologic study estimates the prevalence of itch amongst dermatological patients is around 54.4-88.9% (Dalgard et al., 2020; Weisshaar, 2016). Acute itching affects 8.4% of the population, while chronic type of itch affects from 8 to 38% of the general population (Mollanazar, Koch and Yosipovitch, 2015; Wolkenstein et al., 2003). However, it is essential to study different prevalence estimates of itch with sex, age, ethnicity and geographical location (Mollanazar, Koch and Yosipovitch, 2015).

The overall global prevalence of itch is variable based on the different age groups. For example, pruritus in children is primarily due to AD, which represents between 17 and 22% in highly affected world countries such as United States, Japan, Singapore, and Denmark. The lower prevalence rate is seen in Tanzania (about 7%) and in Turkey (4.3%) (Misery and Ständer, 2010). It is estimated that itch affects about 18% of all pregnant women worldwide (Weisshaar and Dalgard, 2009). In the case of elderly people, only a few studies have investigated the prevalence rate in this age group (Reich, Ständer and Szepietowski, 2011).

Based on the 2006 retrospective study conducted on 4000 elderly patients aged 65 years found that itch was responsible for 11.5% of hospitalizations (Yalçın et al., 2006). This rate increased to 19.5% in the oldest group aged more than 85 years (Yalçın et al., 2006). Interestingly, itch is common among people with lower education levels and low socio-economic status (Ofenloch et al., 2019; Hossenbaccus and Jeewon, 2014). There exists a worldwide variation in the prevalence rate of itch associated with systemic diseases such as hepatic disease, kidney disease, malignancy, iron deficiency, human immunodeficiency virus (HIV) infection, diabetes mellitus, and other endocrine disorders (Tarikci et al., 2015). Amongst the systamic dieseases, skin, kidney, and hepatic diseases are probably the most general causes of itch (Tarikci et al., 2015). Table 1.1 represents the prevalence of itch in the general population in association with common underlying diseases.

Table 1.1 Estimated prevalence of itch in association with common underlying diseases

	Estimated Prevalence	Reference
General population	8.4% (Acute) 8 to 38 % (Chronic)	(Matterne et al., 2011) (Mollanazar, Koch and Yosipovitch, 2015)
Chronic kidney disease	22-90%	(Pisoni et al., 2006) (Patel, Freedman and Yosipovitch, 2007)
Hepatic disease	80–100%	(Bergasa, Mehlman and Jones, 2000)
Depression	17.5%	(Weisshaar, 2016)
Psychiatric diseases	20- 70%	(Schneider et al., 2006)
Pregnancy	18%	(Black et al., 2008) (Weisshaar and Dalgard, 2009)
Post-burn	87%	(Van Loey et al., 2008)
Malignancy	13-19.2 %	(Mollanazar, Koch and Yosipovitch, 2015)
Dermatological diseases		
Psoriasis	67-84%	(Yosipovitch et al., 2000) (Hawro et al., 2020)
Chronic idiopathic urticaria	68-100%	(Weisshaar and Dalgard, 2009) (Patel, Freedman and Yosipovitch, 2007)
Atopic dermatitis	28-91%.	(Chrostowska-Plak et al., 2009) (Martin et al., 2020)
Atopic eczema	87-91%.	(Mollanazar, Koch and Yosipovitch, 2015)
Chronic urticaria	68%	(Yosipovitch et al., 2002)
Infection		
Herpes-Zoster	4- 58%	(Weisshaar and Dalgard, 2009) (Oaklander et al., 2003)
HIV	11-46%	(Singh and Rudikoff, 2003) (Eisman, 2006)
Auto-immune disease		
Multiple sclerosis	20–30%	(El-Baalbaki et al., 2010)
Biliary cirrhosis	18.9-55%	(Talwalkar et al., 2003) (Prince et al., 2002)
Scleroderma	43–45 %	(Haber, Valdes-Rodriguez and Yosipovitch, 2016)
Dermatomyositis	85%	(Goreshi et al., 2011)
Endocrine Disorders		
Diabetes	2.7- 6.5 %	(Neilly et al., 1986)
Hyperthyroidism	60 %	(Yosipovitch, Greaves and McGlone, 2004)
Drug-induced pruritus		
Opioid	2–10% (oral) 10–50% (i.v) 20–100% (i.t)	(Miller and Hagemann, 2011) (Millington et al., 2018)
Chloroquine	60–70%	(Adebayo et al., 1997) (Millington et al., 2018)
hydroxyethyl starch	12.6-54%	(Reich, Ständer and Szepietowski, 2009) (Albegger et al., 1992)

1.1.4 Mechanisms and pathophysiology of itch

In the past few decades, there have been some remarkable improvements in exploring the knowledge about the mechanisms that contribute to itch sensation and the interaction between the PNS and CNS during this episode. Majority of the previous research about itch has been focused on the link between itch and pain. However, literature evidences suggest that itch has several features in common with pain (Davidson and Giesler, 2010). Nonetheless, there is a crucial difference between those types of sensation. For instance, both sensations induce different motor reflexes (pain evokes withdrawal reflex and itch induces scratching or rubbing motor reflex) (Rinaldi, 2019; Paus et al., 2006); opioid analgesic such as morphine inhibits pain sensation, but it can also promote itch in humans and primates (Kjellberg and Tramer, 2001; Lee et al., 2007); sensation of itch can be suppressed by painful stimuli such as mechanical (scratch), thermal, chemical and electrical stimuli (Ward et al., 1996; Nilsson et al., 1997); itch can be induced very locally from superficial skin layers (epidermis) and attached mucous membranes and conjunctiva while most of the human tissue is innervated by nociceptors, and this means pain can be induced by different stimuli in most of the body tissues (Liu and Ji, 2013; Keele, 1970). In spite of all the differences between the two types of sensation, there is evidence of overlapping mediators, functions in primary afferent fibers, as well as spinal cord sensitization, that overlap to a significant degree (Schmelz, 2015).

Interestingly, to understand the mechanism of itch we must understand the mechanism of pain since both pain and itch share approximately the same molecular and neurophysiological mechanisms (Greaves, 1987). Based on previous evidence and many studies, researchers believe that itch sensations are mediated by network interaction between PNS and CNS (Steinhoff et al., 2006). Several peripheral and central mechanisms are generally accepted as possible explanations for the pathophysiology of itch. Despite the fact that the studies in this thesis are focused on the peripheral sensation of itch rather than central neural pathways, I will discuss briefly what is known about both peripheral and central neural pathways in order to provide a more comprehensive picture of how the sensation is provoked and interpreted in the following two sections. Figure 1.2 exhibits a summary of itch proceeding from the skin

to the brain and reveals about the peripheral and central mechanisms contributing to itch.

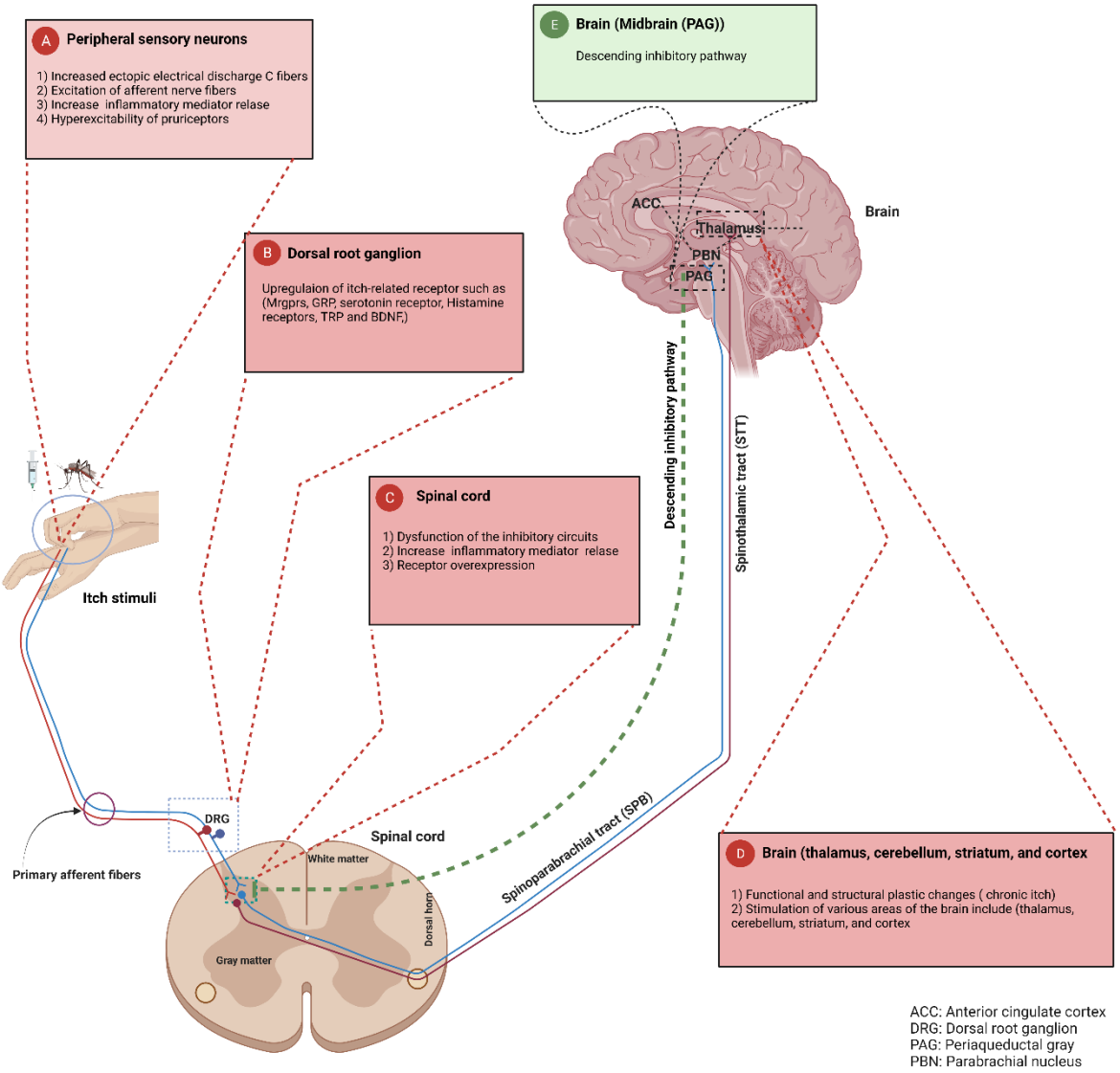


Figure 1.2 Summary of itch proceeding from the skin to the brain. Neural sensitization of itch sensations can be induced by various causes in the skin, dorsal root ganglia, spinal cord, and brain. I tch signals are transmitted from the skin to the spinal cord via peripheral sensory fibres(which are divided into two subsets: histamine-dependent, and histamine-independent pathways), where they are processed by local interneurons before reaching the spinal projection neurons. The itch signals are then transmitted to the brain via the ascending pathways by the spinal projection neurons spinothalamic tract (STT) and the spinoparabrachial pathway (SPB), where they are further processed in a variety of brain areas which may result in central sensitization (adapted from Yosipovitch et al., 2018; Dong and Dong, 2018 and Najafi et al., 2021).

1.1.4.1 Peripheral mechanisms of itch

The skin serves as a protective barrier against any foreign body. Epidermis is the layer that is in direct contact with the environment and it contains primarily keratinocytes and the free nerve ending (Shelley and Arthur, 1957; Joost et al., 2016; Merad et al., 2008; Pasparakis et al., 2014 and Dong and Dong, 2018). The thickest layer of the skin, dermis is located below the epidermis and it contains blood and lymphatic vessels as well as a wide range of immune cells (Dong and Dong, 2018). It consists of nerve endings that transmit various stimuli such as pain, itch, pressure, and temperature (Steinhoff *et al.*, 2006). Within the immune cells, there exists tissues containing mast cells having histamine and other mediators responsible for inflammation (Pasparakis, Haase and Nestle, 2014; Dong and Dong, 2018).

When the skin is directly or indirectly exposed to an irritant substance that leads to activation of skin cells, which in turn causes the release a wide range of peripheral itch mediators including endogenous pruritogens from neuronal (primary sensory afferents neurons) as well as non-neuronal cells (keratinocytes, fibroblast, and local immune cells). The primary sensory neurons are classified into four types (Aα-, Aβ-, Aδ- and C-fibers) based on their signal conduction velocity, diameter, and myelination state of the axons as shown in figure 1.3. (Yam *et al.*, 2018; Julius and Basbaum, 2001). Different sensory information is sent via the different types of primary sensory neurons. Aα fibers sent the proprioceptive information from the skeletal muscles to the spinal cord. Pressure, light and touch information from the mechanoreceptors travels along Aβ fibers. The lightly myelinated Aδ-fibers and the unmyelinated C-fibers have the intermediate and lowest conduction velocities respectively, serve to receive and transmit information relating to itch and pain sensations (Potenzieri and Undem, 2012; McGlone and Reilly, 2010; Misery and Ständer, 2010). The two different types of fibers are involved in the transmission of itch sensation are A-fibers and C-fibers (LaMotte *et al.,* 2014). Aδ-fibers can serve as pruriceptors and nociceptors, while Aβ-fibers serve as nociceptors (LaMotte et al., 2014; Azimi et al., 2016).

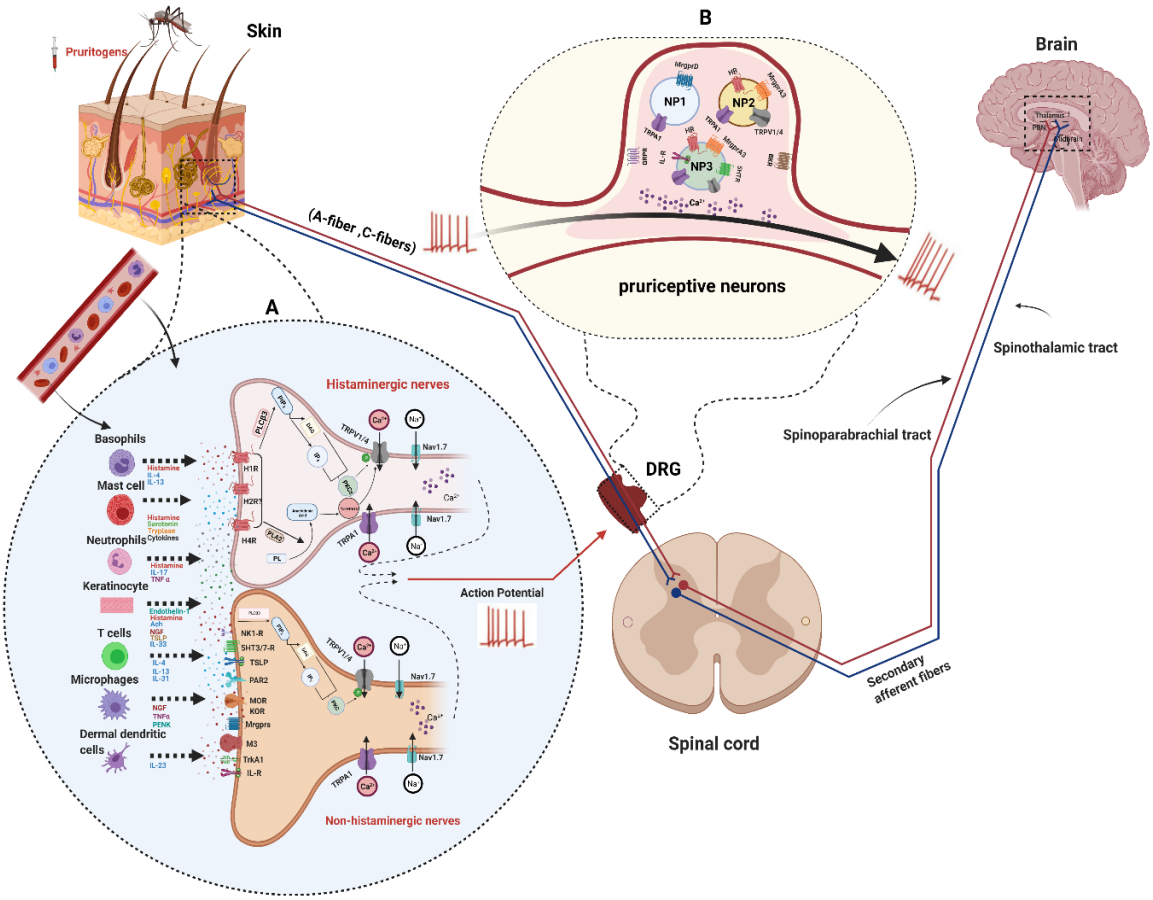
Itch mediators (pruritogens) such as histamine, serotonin proteases, thromboxanes, leukotrienes and IL-4, IL-13, IL-30, and IL-33 lead to the activation of different types of GPCRs receptors such as histamine H1 receptor (H_1R), histamine H4 receptor (H_4R), serotonin receptors(5-HT), protease-activated receptors (PAR2/4), endothelin A receptor (ETA), mas-related G-protein receptor family (Mrgprs), Lysophosphatidic acid receptor (LPAR), bile acid receptor (TGR5), toll-like receptors (TLRs), and cytokines receptors. Figure 1.4. summarising the primary peripheral itch mediators and their receptors involved in itch pathway. Activation of all these receptors expressed in primary sensory neurons results in the activation of phospholipase A2 (PLA_2), phospholipase C-β3 ($PLC\beta_3$) and protein kinase C δ (PCKδ) leading to increase of intracellular Ca^{2+} in primary sensory neurons *via* transient receptor potential subfamily (TRPA1/V1) (Bell, McQueen and Rees, 2004; Liu and Ji, 2013; Kim *et al.*, 2004; Shim *et al.*, 2007). Intracellular Ca^{2+} also increased *via* the PLCβ/inositol 1,4, 5-trisphosphate (IP3)/Ca^{2+} signaling pathway (Liu et al., 2011). Additionally, activation of pruriceptors lead to releases additional itch mediators such as substance P (SP) and tumor necrosis factor (TNF) to induce neurogenic inflammation and potentiate itch signaling (Liu and Ji, 2013; Dong and Dong, 2018; Davidson and Giesler, 2010; Ikoma *et al.*, 2006).

Primary afferent neurons carry itch signals, predominantly small unmyelinated C-fibers with cell bodies located within the dorsal root ganglia (DRG) (Meixiong and Dong, 2017; Dong and Dong, 2018). These neurons transmit itch sensation from the skin to the CNS (Dong and Dong, 2018). The gene expression profiles of the primary afferent neurons can be used to classify the neurons in the DRG (Meixiong and Dong, 2017; Dong and Dong, 2018). The single-cell expression profiling of itch neurons showed three subtypes of itch neurons identified as NP1, NP2, and NP3 (Usoskin et al., 2015; Meixiong and Dong, 2017; Dong and Dong, 2018; Cevikbas and Lerner, 2020). MrgprD is expressed by NP1 neurons, MrgprA3, MrgprC11, H_1R, H_4R, PAR2, is expressed by NP2 neurons, and MrgprA3, H_1R, serotonin receptors, IL-4, IL-13 receptors are expressed by NP3 neurons (Meixiong and Dong, 2017; Dong and Dong, 2018). Notably, all the three subtypes of the neuron are expressed TRP channels (TRPA1, TRPV1), which are critical for itch signaling (Meixiong and Dong, 2017). Over-expression of all these receptors leads to a rise in membrane potential, and activation of the sensory triggers the action potential which

in turn tranfer the sensory information to the CNS (Imamachi et al., 2009; Dong and Dong, 2018).

Myelinated	Type of fiber	Diameter (μm)	Conduction velocity (m/s)	Function
 Myelinated	Aα fibers	12-20	72-120	Light touch Proprioceptors
 Myelinated	Aβ fibers	5-12	36-72	Light touch Proprioceptors
 Thinly myelinated	Aδ fibers	1-5	5-30	light touch Fast pain Nociceptors (thermal, mechanical and chemical)
Unmyelinated	C fibers	0.2-1.5	0.5-2	Slow pain Innocuous temperature Itch Nociceptors (thermal, mechanical and chemical)

Figure 1.3 The classification of sensory fibers based on size, structure, conduction velocity and functions (adapted from Yam et al., 2018; Julius and Basbaum, 2001).



ACC: Anterior cingulate cortex
Bhlhb5: Helix-loop-helix family member B5
BK: Bradykinin
B1/2-R: Bradykinin receptors 1 and 2
Dyn:dynorphin
Gly: Gycine
Glu: gutamate
GRP: Gastrin-releasing peptide
GRPR: Gastrin-releasing peptide receptor
GABA: Gamma-amino butyric acid
His: Histamine
H1R:Histamine 1 receptor
H4R: Histamine 4 receptor
LCN2 :lipocalin-2

IFN-α: Interferons
Mor: Morphine
MOR:μ-opioid receptors
P:Protease
PAG: Periaqueductal gray
PAR2:Protease-activated receptor-2
PMA: premotor area
KOR:κ-opioid receptors
SP: Substance P
SI: Primary somatosensory cortices
SII: Secondary somatosensory cortices
SMA: Supplementary motor area
TRPA: Transient receptor potential ankyrin
TRPV: Transient receptor potential vanilloid
5-HT:Serotonin
5-HT3/7-R: Serotonin receptors type 3 and 7

Figure 1.4 Diagram showing peripheral mediators involved in itch pathway. The skin acts as a protective barrier because it is made up of several layers that are laminated together. **(A)** As soon as an infected or irritated skin area is exposed, the epidermal keratinocytes and the local immune cells (such as mast cells, T-cells, eosinophils, neutrophils, and basophils) release a variety of mediators, such as histamine, serotonin, bradykinen and various proteases (IL-4, IL-13, IL-3 and IL33). Histaminergic nerves and nonhistaminergic nerves are the two subgroups of itch-selective nerve fibers. Receptors for the histamine neurotransmitter (H_1R), (H_2R), and (H_4R) are found in histaminergic nerve fibers and are activated by histamine, while non-histaminergic nerves respond to a variety of pruritogens other than histamine through corresponding receptors and are involved in chronic itch. **(B)** in DRG, according to the expression of GPCRs, itch-sensitive neurons can be classified into three different types. The samll coloured circles represent subtypes of itch nociceptors according to many puplications. These subtypes are named NP1, NP2 and NP3. NP1 neurons express Mrgprd and TRPA (blue). NP2 neurons express histamine recepors, TRPV, MrgprA3 and MrgprC11 (yellow). NP3 neurons express 5-HTR, ILR, TRPV, MrgprA3 and MrgprC11 (green).(adapted from Leslie, 2013; Green and Dong, 2016; Meixiong and Dong, 2017; Dong and Dong, 2018).

1.1.4.2 Central mechanisms of itch

During the past 20 years, much more information has become available on the role of the different neuronal receptors present in the spinal cord that contributes to the process associated with itch. After peripheral sensitization, the signal and action potentials travelling from at least two types of peripheral C-fibers to the dorsal horn of the spinal cord (Potenzieri and Undem, 2012). In the dorsal horn of the spinal cord, the primary afferent nerves synapse to secondary order nerve fibers (Dong and Dong, 2018). In the synapse between the primary-order neurons and second-order neurons that stimulates the release of neurotransmitter and neuropeptides at the spinal cord dorsal horn level (lamina I) (Liu and Ji, 2013). Recently, several studies have indicated that numerous neural receptors in the spinal cord have been shown to be involved in itch transmission such as opioid receptors (Ballantyne, Loach and Carr, 1988), gastrin-releasing peptide (GRP) receptors (Sun and Chen, 2007), neurokinin-1 receptor (Ständer *et al.*, 2010), glutamate receptors (Cevikbas *et al.,* 2011), bradykinin receptors (Costa *et al.*, 2010), 5-HT receptors (Hägermark, 1992; Cevikbas *et al.,* 2011)and histamine receptors (Andrew and Craig, 2001). Table 1.2 consists of the collected information obtained from the publications about spinal neurotransmitters, receptors, and their functional relevance to itch.

Along with the other receptors and mediators, GRP is a critical mediator of the spinal itch transmitter, which is released either from the central primary sensory neurons or from local spinal neurons (Sun and Chen, 2007; Lee et al., 2016). Recentally, several stuides demonstrated an essential role for itch transmission for GRP/ GRPR- expressing neurons in the spinal cord (Sun and Chen, 2007; Liu et al., 2011; Lee et al., 2016). GRPR is expressed in laminae I and II superficial dorsal horn neurons (Liu et al., 2011; Lee et al., 2016). Interstnigly, GRPR mutant mice exhibit decreased scratching in response to different type of pruritic agents, particularly non-histaminergic pruritogens (Liu et al., 2011). Notably, inhibition of GRPR+ neurons in the dorsal horn significantly decreased scratching induced by both histaminergic and non-histaminergic pruritogens (Sun et al., 2009). On the other hand, pain transmmtion did not affected by inhibition of $GRPR^{+}$ neurons (Sun et al., 2009; Lee et al., 2016).

Continued release of neurotransmitters lead to increased intrinsic excitability of second-order neurons in the spinal cord (Costigan et al., 2009) and subsequent activation of the N-methyl-D-aspartate (NMDA) receptors, which increase the intracellular Ca^{2+} levels across the cord *via* the anterior commissure and ascending along the contralateral spinothalamic tract to the laminar nuclei of the contralateral thalamus (Costigan et al., 2009). Figure 1.5 summarising the spinal itch mediators and their receptors.

In the brain, the itch signals ascend to the brain *via* two pathways following the excitability of second-order neurons in the spinal cord that includes the path through the spinothalamic tract towards the thalamus and via the spinoparabrachial pathway towards the parabrachial nucleus in the brain stem (Mu *et al.*, 2017; Yosipovitch, Rosen and Hashimoto, 2018). Interestigly, itch experience is not localized to one brain area, and induction of itch leads to activations of multiple brain regions (Jovanović, 2014; Mochizuki et al., 2019). Several previous studies using brain imaging have reported that histamine or cowhage can provoke activation of multiple brain areas such as thalamus, cerebellum, striatum, prefrontal cortex (PFC), premotor cortex (PM) , primary motor cortex (MI), anterior and posterior cingulate cortex, primary (SI) and secondary (SII) somatosensory cortex, precuneus hippocampus, hypothalamus, amygdala and midbrain (Hsieh *et al.*, 1994; Papoiu *et al.*, 2012; Mochizuki and Kakigi, 2015; Ikoma *et al.*, 2011). In clinical study by Pfab et al., in 2012, among healthy volunteers, it was found that histamine-induced itch activated a spread neural network in the brain, whereas cowhage-induced histamine-independent itch activated certain areas of the brain including insula, thalamus, and putamen (Pfab et al., in 2012).

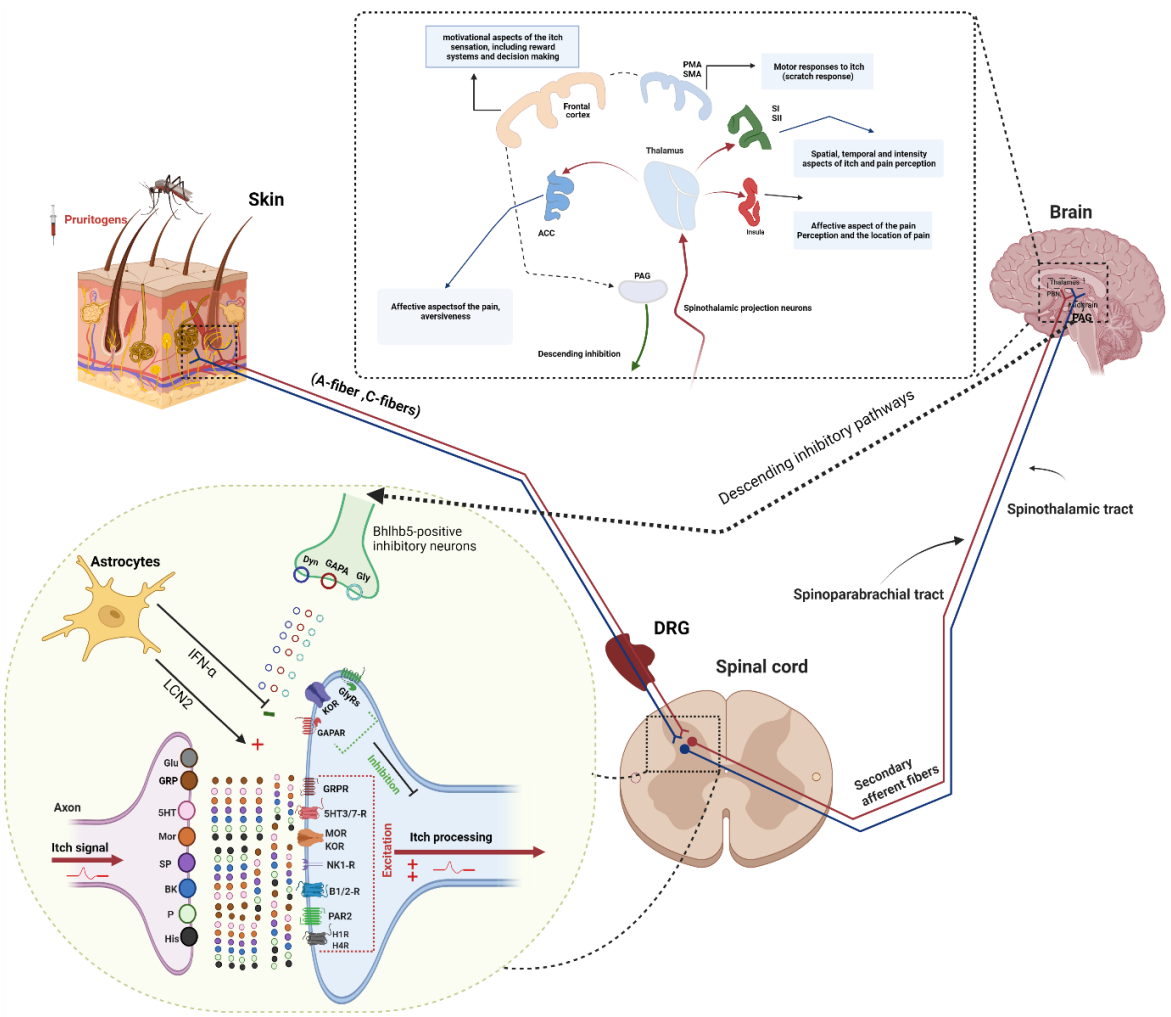
It is also important to mention the descending inhibitory pathway that occurs after itch stimuli. This inhibitory pathways are originating in supraspinal areas in the brain which activate helixloop-helix family member B5 (Bhlhb5)-positive neurons and modulate the sensation of itch (Ross et al., 2010; Yosipovitch et al., 2018). This negative control of itch is medated by excitatory α-adrenoceptors on Bhlhb5-positive inhibitory neurons, as well as central terminals of primary sensory neurons in the dorsal horn (Gotoh et al., 2011; Yosipovitch et al., 2018). Attenuation of the itch sensation can be achieved through mechanical stimulation, pain, and cooling (Yosipovitch et al., 2007). These sensations

cause the activation of Bhlhb5–positive inhibitory neurons in the spinal cord (Yosipovitch et al., 2018). These Inhibitory interneurons release neurotransmitters such as opioid peptide dynorphin, glycine, and gamma-amino butyric acid (GABA), which then exert their inhibitory effect on GPRP-positive neurons (Ross et al., 2010; Braz et al., 2014; Huang et al., 2018; Yosipovitch et al., 2018).

Table 1.2 Spinal receptors involved in itch.

Neurotransmitter	Receptor	Functional relevance to itch induction	Reference
opioid receptors	μ-opioid receptor (MOR) κ-opioid receptor (KOR)	Spinal and epidural administration of morphine frequently causes segmental itch. μ-opioid receptor antagonists, such as naloxone and naltrexone, have anti-pruritic effects, κ-opioid receptor agonist such as Nalfurafine, a, is effective in control itch in haemodialysis patients	(Ballantyne, Loach and Carr, 1988) (Mikuni *et al.*, 2010) (Phan *et al.*, 2010) (Wikström *et al.*, 2005)
Gastrin-releasing peptide	BB-2	Intrathecal administration of BB2 antagonists suppresses pruritogenic scratching behavior. BB2-knockout mice scratch less when pruritogens such as compound 48/80 or chloroquine are used to induce itching.	(Sun and Chen, 2007) (Sun *et al.*, 2009) (Handwerker and Schmelz, 2009)
Substance P	NK1R	Intracisternal microinjections of NK1R antagonist supporting a role for substance P in itch	(Ständer *et al.*, 2010) (Carstens *et al.*, 2010)
Bradykinin and Proteases (trypsin, tryptase, cathepsins, and kallikreins)	PAR2 B1/2-R	Intrathecal administration of antagonists of bradykinin receptors decreased scratching induced by a PAR2 agonist. Bradykinin receptors have been indicated to play a role in PAR2-mediated itch at the spinal cord level	(Akiyama et al., 2009) (Costa *et al.*, 2010)
Glutamate receptors	NMDA AMPA	NMDA receptor antagonists may be effective in inhibiting itch. Gabapentin and pregabalin, both of which inhibit glutamate release by decreasing Ca2+ influx, are effective for itch. Intrathecally administered NMDA agonists such as spermine produces the scratching behaviour in mice Glutamate activates the AMPA receptor, a likely candidate for itch transmission	(Tan-No *et al.*, 2000) (Cevikbas et al., 2011) (Koga *et al.*, 2011)
5-HT receptors	5HT3-R and 5HT7-R	Systemically administration of 5-HT3 receptor antagonist, reduces opioid-induced itch in the dorsal horn of the spinal cord. Local injection of serotonin exerts a prolonged activation of the superficial dorsal horn neurons that are also responsive to noxious stimuli	(Bonnet *et al.*, 2008)
Histamine receptors	H_1R, H_2R,H_3R and H_4R	Morphine's antinociceptive effects are enhanced in H_1R, H_2R, or H_3R deficient mice, indicating that these receptors may play a role in morphine-induced itch.	(Andrew and Craig, 2001) (Cevikbas et al., 2011)

AMPA =α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
protease-activated receptor-2 (PAR)2
BB2- = Bombesin receptor-2
NK1R= neurokinin-1 receptor
NMDA= N-methyl-D-aspartate



ACC: Anterior cingulate cortex	IFN-α: Interferons
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BK: Bradykinin	MOR:μ-opioid receptors
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Dyn:dynorphin	PAG: Periaqueductal gray
Gly: Gycine	PAR2:Protease-activated receptor-2
Glu: gutamate	PMA: premotor area
GRP: Gastrin-releasing peptide	KOR:κ-opioid receptors
GRPR: Gastrin-releasing peptide receptor	SP: Substance P
GABA: Gamma-amino butyric acid	SI: Primary somatosensory cortices
His: Histamine	SII: Secondary somatosensory cortices
H1R:Histamine 1 receptor	SMA: Supplementary motor area
H4R: Histamine 4 receptor	5-HT:Serotonin
LCN2 :lipocalin-2	5-HT3/7-R: Serotonin receptors type 3 and 7

Figure 1.5 Diagram showing spinal receptors involved in itch pathway (adapted from Yosipovitch et al., 2018; Cevikbas and Lerner, 2020).

1.1.5 Treatment options for itch

The management of pruritus is one of the challenges faced by both patients and health care professionals. The efficacy of traditional systemic therapy is limited and new treatments have been slowed down from the development (Summey Jr and Yosipovitch, 2005). Generally, treatment for the itch is based on its aetiologies and the primary cause of the illness (Rinaldi, 2019). The treatment of acute itch is usually simple and straightforward, however the management of the chronic type of itch can be much more complex and time-consuming (Leslie, 2013). The first step in the management of itch is to identify whether it is caused by any dermatological disease or due to systemic disorders (Cowan and Yosipovitch, 2015). Because of the complicated pathophysiology of itch and its effect on individual's daily activities and quality of life, there is a need for the doctors to understand and address various aspects of itch for each patient (Hong *et al.*, 2011). These aspects include: (1) identifying and addressing the trigger factors such as sweating, exogenous factors, aeroallergens, and food allergies; (2) start symptomatic therapy until the final diagnosis is confirmed; (3) addressing psychological, cognitive, and behavioral components; (4) personal and/or family history; and (4) educating the patient (Hong *et al.*, 2011; Steinhoff *et al.*, 2011). A recommendation by the British Association of Dermatology suggests that initial blood checks involving complete blood cell count, iron content checks, thyroid function checks, checking of urea and electrolyte levels, hepatic function tests and screening of autoimmune diseases is necessary. It introduces potential therapeutic ladders that form the basis to explore effective therapeutic itch strategies in numerous diseases.

Generally, the current international guidelines for the management of pruritus in many countries recommend a step-by-step treatment approach (Ständer *et al.*, 2006; Metz, Grundmann and Ständer, 2011). This approach is based on the patient's age, sex, medical condition, medication history, the severity of symptoms, intensity, and localization of itch (Weisshaar *et al.*, 2012; Metz, Grundmann and Ständer, 2011). Indeed, the patient should be fully involved in their treatment plan in order to ensure compliance, which results in more successful itch management (Weisshaar *et al.*, 2012; Leslie, Greaves and Yosipovitch, 2015).

Topical therapies are still a fundamental part of the management of itch. They are considered as first-line therapy for an acute and mild type of itch, mainly where systemic therapies are contraindicated or cannot be tolerated (Leslie, Greaves and Yosipovitch, 2015; Hong *et al.*, 2011). Indeed, topical therapies commonly have a minimum side effects comparing to systemic medications (Leslie, Greaves and Yosipovitch, 2015). According to many clinical studies topical therapies in the management of itch are divided into 4 types: (1) emollients and moisturizers; (2) anti-inflammatory agents such as corticosteroids, salicylates, calcineurin-inhibitors, and coal tar products; (3) antihistamines; (4) phototherapy (UVA, UVA1, UVB, and PUVA); and (5) miscellaneous anti-pruritic agents such as menthol, calamine, camphor, urea, local anaesthetics, methylene blue, topical doxepin, and topical naltrexone and all these do not target inflammation and immune activation directly (Hong et al., 2011; Misery and Ständer, 2010; Leslie, Greaves and Yosipovitch, 2015). Recently, many preclinical studies on the pathophysiology and mechanisms of itch have identified specific receptors agonists and antagonists targeting a wide range of local itch mediated receptors such as proteases, neuropeptides, histamine, cytokines, and opioids (Leslie, Greaves and Yosipovitch, 2015; Leslie, 2013; Hong *et al.*, 2011).

To provide a generalized treatment to those with a chronic itch, a systemic treatment plan is needed to manage these cases. Several of these drugs would target local or central pathophysiology to fulfil their anti-pruritic effects. Generally, it takes a long time to control the chronic type of itch and in most clinical cases, combination therapies are needed with different mechanisms of actions (Hong et al., 2011). The first-line systemic therapy includes oral antihistamines, which have traditionally been believed to be the mainstay of itch management, particularly 1st generation antihistamines that are sedative, help the patient sleep at night and therefore interrupt and break the itch-scratching cycle (Leslie, Greaves and Yosipovitch, 2015). Itch can be treated systemically using immunosuppressants, phototherapy, anticonvulsants, antidepressants, and μ-opioid antagonists and κ-opioid agonists. Table 1.3 and 1.4 present the most common topical and systemic treatments for different types of itch.

Recently, researchers have shown increased attention to itch management. This new emphasis on itch as a specific target has led to the curiosity in finding novel drugs like

histamine H_4R antagonists and at present, they are in clinical studies (Dunford et al., 2007). Therefore, it is expected that further drugs that target specific itch receptors innvolved in itch will come into the market in the future. Accordingly, one of the goal of this PhD project was to study the peripheral mechanisms of itch in order to understand underline mechanisms affecting transmission of itch, with the hope of identifying new approaches to itch treatment.

Table 1.3 Common topical treatments for pruritus

Agent	Mechanism of action	Indications	Reference
Emollients and Moisturizers	Improves skin barrier function, membrane fluidity and cell signaling	Itch associated with: Xerosis, Atopic dermatitis (AD), Allergic contact dermatitis (ACD), Psoriasis	(Elmariah and Lerner, 2011) (Purnamawati *et al.*, 2017)
Corticosteroids	Inhibit inflammatory cytokines release and reduce the local inflammation	Pruritus associated with: AD, ACD, Psoriasis, Prurigo nodularis (PN) and Lichen simplex chronicus (LSC)	(Elmariah and Lerner, 2011) (Kircik, 2011) (Zhai *et al.*, 2000) (Leslie, Greaves and Yosipovitch, 2015)
Calcineurin Inhibitors	Immunomodulators regulate T-cell activation and inhibit the release of inflammatory cytokines	Pruritus associated with: AD, PN, rosacea, lichen sclerosis and anogenital pruritus	(Elmariah and Lerner, 2011) (Leslie, Greaves and Yosipovitch, 2015) (Pavlis and Yosipovitch, 2018) (Hong *et al.*, 2011)
Antihistamines	Block histamine 1 and/or 2 receptors	Pruritus associated with :AD, ACD , LSC and nummular dermatitis	(Eschler and Klein, 2010) (Elmariah and Lerner, 2011) (Leslie, Greaves and Yosipovitch, 2015) (Pavlis and Yosipovitch, 2018)
Naltrexone	μ-opioid receptor antagonists	Pruritus associated with: Chronic urticaria and AD	(Bigliardi *et al.*, 2007) (Hong *et al.*, 2011)
Capsaicin	Desensitization of TRPV1-expressing cutaneous on C-sensory fibers, depleting substance P over time and inhibit neural transmission	Atopic eczema, brachioradial pruritus, Neuropathic itch , Uremic pruritus, PN, and aquagenic pruritus.	(Weisshaar *et al.*, 1998) (Leslie, Greaves and Yosipovitch, 2015)
Vitamin D3 or its analogues	Inhibiting TNF-α mRNA expression and influences keratinocyte proliferation and differentiation	Pruritus associated with psoriasis and PN	(Katayama, Miyazaki and NISHIKA, 1996) (Kircik, 2009)
Anaesthetics	Sodium channel blocker	Neuropathic itch and AD	(Young *et al.*, 2009) (Shuttleworth *et al.*, 1988) (Hong *et al.*, 2011)

Table 1.4 Common systemic treatments for pruritus

Agent	Mechanism of action	Indications	Reference
Antihistamines	Block histamine 1 and/or 2 receptors	Itch associated with: Xerosis, Atopic dermatitis (AD), Allergic contact dermatitis (ACD) and Insect bite reactions	(Uehara, 1982) (Hong *et al.*, 2011) (Leslie, Greaves and Yosipovitch, 2015)
Anticonvulsants	Inhibit spinal mechanisms in the transmission of the perception of itch	Pruritus of cholestasis, Neuropathic itch, Uremic pruritus, Prurigo nodularis and Postburn pruritus	(Bergasa *et al.*, 2006) (Metz, Grundmann and Ständer, 2011) (Leslie, Greaves and Yosipovitch, 2015)
Opioid receptor agonists	μ -opioid and k-opioid receptor antagonists	Pruritus of cholestasis, AD and chronic urticaria	(Maurer, Poncelet and Berger, 2004) (Pavlis and Yosipovitch, 2018)
Antidepressants	selective monoamine reuptake inhibition	Itch associated with: advanced cancer, solid carcinoma leukaemia, lymphoma, chronic kidney disease, cholestasis and atopic dermatitis	(Hundley and Yosipovitch, 2004)
Serlopitant	Neurokinin-1 receptor antagonist	PN and chronic itch	(Leslie, Greaves and Yosipovitch, 2015) (Maurer, Poncelet and Berger, 2004) (Pavlis and Yosipovitch, 2018)
Corticosteroids	Inhibit inflammatory cytokines release and reduce the local inflammation	Pruritus associated with : Atopic dermatitis (AD), Allergic contact dermatitis (ACD),and Psoriasis	(Schmitt *et al.*, 2010) (Maurer, Poncelet and Berger, 2004) (Pavlis and Yosipovitch, 2018)
Thalidomide	inhibit the production of interleukin (IL)-6	PN and Postburn pruritus	(Maurer, Poncelet and Berger, 2004)

1.1.6 Animal models of itch

Over the last century, scientists and itch researchers have developed various methods for evaluating itch in animal and human subjects to shed light on some of the most critical pathways mediating this unpleasant sensation. Generally, aminal models of itch are essential tools to understand the underlying mechanisms and develop safe and effective treatment options (Kuraishi, 2015; Hoeck et al., 2016). Furthermore, it would be challenging to study and understand the pathophysiology of itch at the molecular level if we did not have these tools. Therefore, many animal models of itch have been developed and characterised over the past two decades to simulate the clinical future of itch with a variety of aetiologies in the future (Andoh and Kuraishi, 2007). In the literature, numerous studies describe various animal models for assessing the pathophysiology of acute and chronic itch. Table1.5 summarizes the most common acute and chronic itch models.

Most itch models are based on local administration of pruritogens intradermally (i.d.) or subcutaneously (s.c.) in different types of animals such as mice, rats, guinea pigs, and monkies. However, another route can be used, such as applying pruritogens to the ear, eye or abdomen (Hoeck et al., 2016; Yuan et al., 2016). The rostral back model is the most widely used model to test and evaluate the anti-itching properties of the compounds. This model involves injecting pruritogens locally (i.d. or s.c.) into rodents' rostral backs and quantifying hind paw scratching bouts toward the injection site (Obara et al., 2011; Shimada et al., 2006). Other routes of injection can be used as well, such as rodents are injected with pruritogens either s.c. or i.d. into in various other locations such as hind paw (Hagiwara et al., 1999), calf (LaMotte et al., 2011), cheek (Shimada and LaMotte, 2008) or in legs (LaMotte et al., 2011). These models differ in terms of how symptoms are induced, but they are all based on observations of hind limb scratching (Pereira and Misery, 2010). Some of these models were used to examine acute types of itch, while others were used to study chronic types (Hoeck et al., 2016; Yuan et al., 2016). In this thesis, I used the rostral back model of an acute itch to investigate the role of peripheral histamine and its receptors and evaluated the anti-pruritic efficacy of two novel ligands targeting the peripheral histamine system in acute itch in mice.

Figure 1.6. shows the difference between normal responses and itching responses. Mice are the most frequently used rodents in the rodent family for studying the physiological pathway of itch in the peripheral (Andoh, 2006; Shimada et al., 2006) or the central nervous system (Sun and Chen, 2007). Mice with induced diseases could be used to clarify the underlying mechanisms and develop new and effective therapies for itch associated with local or systemic disorders such as acute itch, atopic dermatitis, autoimmune skin diseases and cholestasis-associated pruritus (Umeuchi et al., 2005; Nelson et al., 2006; Yatsuzuka et al., 2007; Voisin et al., 2021).

As with any aminal model, there are several comprehensive reviews available that discuss the limitations and drawbacks of conventional animal models of itch in great detail (Shimada and LaMotte, 2008; Pereira and Misery, 2010; Bell, 2004). Despite its critics, animal models of itch will remain valuable tools for addressing questions about itch mechanisms and advancing our understanding of how this sensation occurs, progresses, and can be controlled and eliminated.

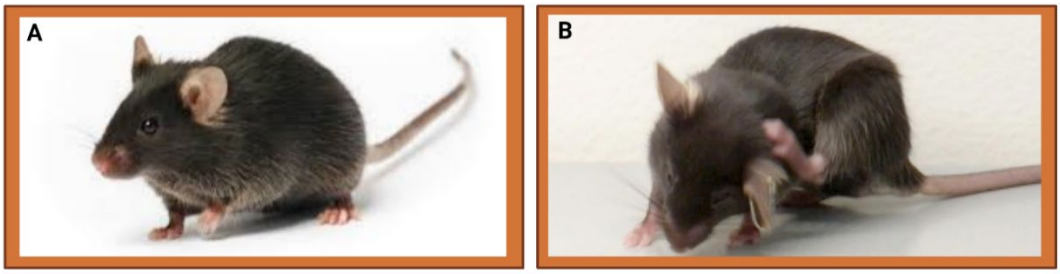


Figure 1.6 Rostral back model of acute itch. In this model pruritogens are injected s.c. or i.d. into the rostral back of mice, and itch is measured by quantification of hind paw scratching bouts towards the injection site. **(A)** Control mouse without itching and **(B)** Mouse showing itching responses.

Table 1.5 Most common acute and chronic itch models

Model name	Type of pruritogens	Animal	Mechanism	Route of administration	Reference
Animal models of acute itch					
Intradermal or subcutaneous injection of pruritogens	**Histamine**	Rats and mice	H_1R and H_4R	Rostral back Rostral back Rostral back Rostral back Cheek Cheek	(Inagaki et al., 1999) (Ohtsuka et al., 2001) (Akiyama et al., 2012) (Akiyama et al., 2014) (Shimada and LaMotte, 2008) (Akiyama et al., 2010)
	Compound 48/80	Mice	Mast cell degranulation	Rostral back Rostral back Rostral back Rostral back Rostral back	(Inagaki et al., 1999) (Kuraishi et al., 1995) (Orito et al., 2004) (Chitme et al., 2010) (Zhao et al., 2013)
	Substance P	Rats and mice	Mast cell degranulation and NK1R	Rostral back Rostral back Rostral back Cheek	(Kuraishi et al., 1995) (Andoh and Kuraishi, 2000) (Andoh and Kuraishi, 2002) (Akiyama et al., 2010)
	Serotonin	Rats and mice	5-HT2, 5-HT3 and 5HT-7	Rostral back Rostral back Rostral back Hind paw	(Andoh and Kuraishi, 2000) (Jinks and Carstens, 2002) (Akiyama et al., 2012) (Hagiwara et al., 1999)
	Chloroquine	Mice	MrgprA3/ MrgprX1	Rostral back Rostral back Rostral back	(Liu et al., 2009) (Akiyama et al., 2012) (Akiyama et al., 2014)
	BAM8-22	Mice	MrgprC11/ MrgprX1	Rostral back Rostral back	(Akiyama et al., 2012) (Liu et al., 2009)
	β-alanine	Mice	MrgprD	Rostral back and cheek Rostral back	(Liu et al., 2012) (Liu et al., 2009)
	Mucunain	Mice Monkeys	PAR-2/ PAR-4	Cheek Lower leg	(Akiyama et al., 2010) (Johanek et al., 2008)
	Leukotriene	Mice	BLT1 receptor	Rostral back Rostral back	(Andoh and Kuraishi, 2000) (Andoh and Kuraishi, 2002)
Applying eye drops	**Histamine**	Mice	H_1R and H_4R	Eye	(Nakano et al. 2009)
	Leukotriene	Mice	BLT4 receptor	Eye	(Andoh et al., 2012)

Animal models of chronic itch					
Dry skin pruritus	**Repeated cutaneous application of an acetone/ether (1:1) solution**	Mice Rats Mice Mice	Dry skin pruritus	Rostral back Rostral back Hind paw Cheek	(Miyamoto et al., 2002) (Nojima et al., 2003) (Akiyama et al., 2010) (Valtcheva et al., 2015)
Contact hypersensitivity (CHS)	**Repeated application of haptens, Oxazolone or 2,4,6-Trinitro-1-chlorobenzen (TNCB)**	Mice Mice Mice	H_1R Opioid-receptor MrgprA3 and MrgprD IL-1β, 4, 6 and 18	Ear Rostral back Abdominal skin Ear	(Tamura et al., 2005) (Tsukumo et al., 2010) (Qu et al., 2014) (Harada et al., 2005)
Genetic models of itch	**IL-18-deficient mice and IL-1-deficient mice** **$TRPV3^{Gly573Ser}$ transgenic mice**	Mice Mice	IL-1and 18 TRPV3		(Konishi et al., 2002) (Yoshioka et al., 2009)

1.2 Neuropathic Pain

1.2.1 Definitions and background

Acute pain is a natural protective mechanism that protects the bodies of humans and animals from serious internal damage due to injury (Basbaum et al., 2009). In other terms, the sensation of pain gives the body an early alarm about a potentially harmful stimulus (Woolf and Salter, 2000). In 1968, H. Merskey defined pain as *"an unpleasant experience which we primarily associate with tissue damage or described in terms of such damage, or both"* (Merskey, 1968). In 1979, the International Association for the Study of Pain (IASP) rephrased this definition to *" an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage''*. As in the latest 2020, IASP described the pain as *"an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage"* (Olivia Sonneborn et al., 2020). The new 2020 IASP definition has been amended and modified further to become broader to include several aspects like individuals suffering from pain but cannot explain or describe it, such as infants, elderly, intubated or critical care patient, individuals with cognitive or mental disabilities, besides this the new definition of pain is also included of animals along with humans (Olivia Sonneborn et al., 2020).

In contrast to acute pain, chronic pain is one of the most complex and distressing public health problems, as it produces a significant socio-economic burden (Dueñas et al., 2016). Chronic pain has a negative impact on the patient's perceptions and emotion including its impact on their families, caregivers, and social circle (Ojeda et al., 2014; Dueñas et al., 2016).

Chronic pain can generally be classified into three major types: nociceptive, inflammatory, and neuropathic (Jensen and Hansson, 2006). Neuropathic pain appears to have greater intensity, longer duration, and a greater possibility of non-recovery than other types of chronic pain (Nickel, Seifert and Maihöfner, 2012; Torrance *et al.*, 2006). It refers to any pain originating from a primary injury or damage in the somatosensory system (Campbell and Meyer, 2006; Zimmermann, 2001). This damage can arise as a result of injury, trauma, or might be due to the side effects of certain medicines (Yu et

al., 2013). Indeed, it can occur along with other common diseases, such as; diabetes, syphilis, and multiple sclerosis (Mendlik and Uritsky, 2015). In 1994, neuropathic pain was initially defined by IASP as *"pain initiated or caused by a primary lesion or dysfunction in the nervous system"* (Merskey and Bogduk 1994). However, many researchers have not widely accepted this definition of neuropathic pain, primarily because the expression ''dysfunction'' is very general and does not specify what falls under the banner of this term (Magrinelli, Zanette and Tamburin, 2013; Bouhassira, 2019). Fifteen years later, to overcome the limitations in the definition of neuropathic pain, the Neuropathic Pain Special Interest Group (NeuPSIG) of the IASP modified and redefined it as *"pain arising as a direct consequence of a lesion or disease affecting the somatosensory system"* (Treede *et al.*, 2008). The updated definition is widely accepted because it incorporates the multidimensional pain experience, including peripheral afferent fibres (Aβ, Aδ, and C fibres) and central neurons (Colloca *et al.*, 2017). It included the somatosensory system which is part of the sensory nervous system responsible for various processes including; the perception of touch, pain, pressure, movement, body position, flutter and temperature (Kaas, 2004; Colloca *et al.*, 2017).

Neuropathic pain is a common condition that is caused due to different aetiologies and can be grouped into either peripheral or central neuropathic pain syndromes (Brooks and Kessler, 2017). Peripheral neuropathic pain is initiated by injury or damage to the peripheral nervous system, whereas central neuropathic pain occurs due to central lesions or diseases to the central nervous system (Meacham et al., 2017; Brooks and Kessler, 2017). Neuropathic pain is a complex, multifactorial condition and the exact underlying mechanism is not fully understood (Perdue, 2019) with many different signs and symptoms, including different aetiology, causes, and anatomical distribution (Jensen *et al.*, 2001). Neuropathic pain is typically characterised by spontaneous pain, hyperalgesia (increased sensitivity to harmful stimulation around the site of injury), allodynia (pain due to a normally harmless stimulus), and causalgia (constant burning pain), as well as by paresthesias (abnormal sensations, such as 'pins and needles', tingling, prickling, reduced or even loss of sensation), dysesthesias (altered perception of sensation with an abnormal feeling associated with stimulation, such as touching over the affected area causes 'strange feeling), and in some cases impairment of normal

sensation (Campbell and Meyer, 2006; Haanpää and Treede, 2010). Indeed, it is mostly associated with the combination of positive and negative signs and symptoms (Colloca *et al.*, 2017; Magrinelli, Zanette and Tamburin, 2013). Positive signs and symptoms indicate gain-of-function of the somatosensory system such as paresthesias, spontaneous pain, increased sensation of pain. Negative signs and symptoms indicate loss of function of the somatosensory system such as hypoalgesia and hypoaesthesia (Baron, Binder and Wasner, 2010; Colloca *et al.*, 2017). Figure 1.7 encapsulates the vital features of positive and negative signs and symptoms of neuropathic pain and some important definition of neuropathic pain signs and symptoms according to IASP. However, it is important to note that the signs and symptom of chronic pain, in particular, neuropathic pain does not present the same challenges for all patient, and sometimes it may have an effect on diseases duration (Jensen and Baron, 2003).

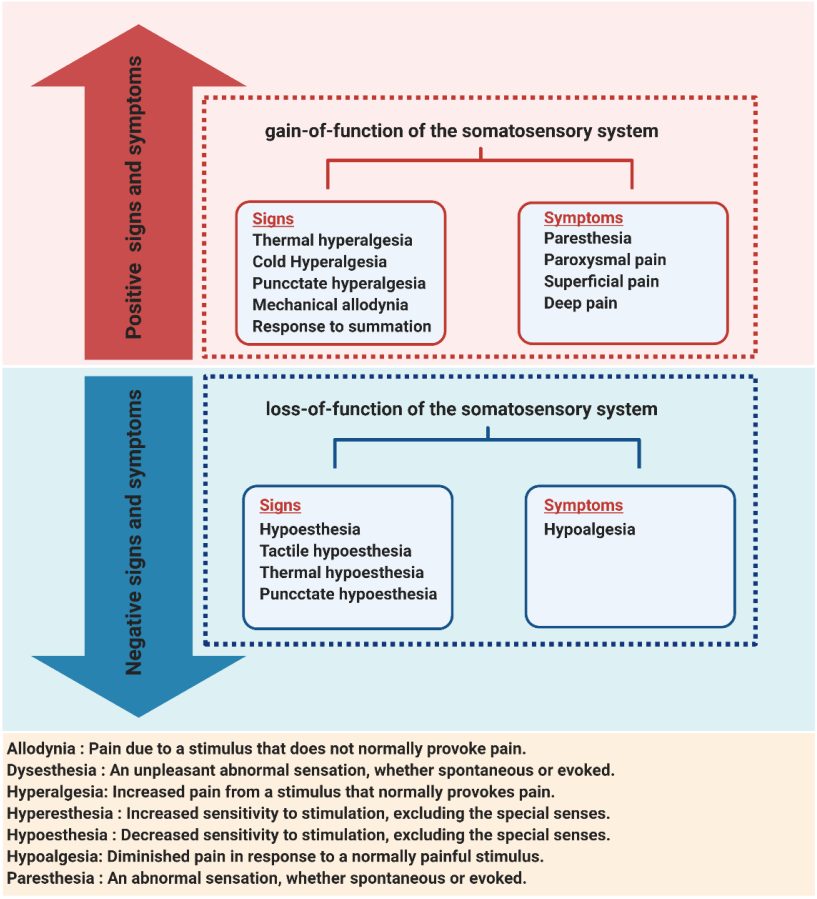


Figure 1.7 A summary of the key features of both positive and negative signs and symptoms of neuropathic pain. The negative signs and symptoms indicate loss-of-function of the somatosensory system while, positive signs and symptoms indicate gain-of-function of the somatosensory system (adapted from Baron, Binder and Wasner, 2010; Colloca et al., 2017).

Clinically neuropathic pain is observed as a contentious pain and/or evoked pain, and is expressed in numerous ways, such as tingling, jabbing, burning, shooting, spasm, prickling, electric shock-like, squeezing or cold (Rice et al., 2018; Xie, 2011).

Neuropathic pain seems to have a significant negative impact on health-related quality of life and daily functioning, including; physical, emotional, social, and well-being (Jensen, Chodroff and Dworkin, 2007). Patients also seem to suffer greater pain intensity compared to other pain types, such as nociceptive pain (Smith et al., 2007). Patients with neuropathic pain also appear to have an impairment of the ability to initiate or maintain sleep, leading to change in mood and development of behavioural abnormalities, such as stress, sleep disturbances, anxiety, and depression (McCarberg and Billington, 2006).

Like many patients with chronic pain conditions, neuropathic pain patients use a high proportion of medical care resources, such as several visits to medical specialists and number of days of hospitalization, use of medications, emergencies, rehabilitation and physiotherapy sessions (Colloca *et al.*, 2017; Berger, Dukes and Oster, 2004; Schaefer *et al.*, 2014). Indeed, a substantial amount of information has been recently published about the relationship between neuropathic pain and healthcare costs (Ruiz-Negrón *et al.*, 2019; Colloca *et al.*, 2017). For example, United States assessed that the health system cost for treatment of chronic pain disorder more than $600 billion yearly, which is much greater compared to the annual costs of other chronic diseases such as cardiac disease, cancer, and diabetes mellitus (Gaskin and Richard, 2012; Machado-Duque et al., 2020). A recent clinical European study of approximately 4,000 patients reported an increase in both direct costs (number of consultations, prescribed drug treatment, and surgical procedures), as well as indirect costs (work and productivity loss) (Liedgens et al., 2016). The findings of this study concluded that the annual total costs, which include both direct and indirect cost per neuropathic pain patient, were € 9,305 in Italy, €9,685 in the UK, €10,313 in France, €10,597 in Spain, and €14,446 in Germany (Liedgens et al., 2016).

The management of neuropathic pain is still one of the major challenges faced by healthcare organizations. Unfortunately, the existing evidence suggests that the response to existing treatment options is often insufficient and majority of patients do not achieve satisfactory pain relief (Dworkin et al., 2001). Consequently, there is a considerable need to explore novel pharmacotherapies for the successful treatment of neuropathic pain; therefore, this requires a better understanding of the mechanisms involved in neuropathic pain and more appropriate tools for assessing chronic pain sufferers. My work, presented here may suggest that selective targeting of the histaminergic system could potentially interact with mechanisms that underly this type of pain. This may potentially represent a viable therapeutic target for the management of the neuropathic pain states.

1.2.2 Classification of neuropathic pain

Classification of neuropathic pain remains a topic of controversy. There is no universally accepted classification system for this type of pain. Recent studies demonstrated that universal classification systems that are based on different overviews including more understanding of the condition are being researched and developed, encompassing symptoms, aetiology, pathophysiology, location and pain distribution (Haanpää and Treede, 2010; Colloca *et al.*, 2017; Treede *et al.*, 2008). In clinical practice, the most common classification system for neuropathic pain is based on the underlying aetiology, the anatomical location of the specific lesion, based on symptoms and signs or basis of pharmacological agents (Jensen *et al.*, 2001; Sindrup and Jensen, 1999; Jensen and Baron, 2003).The anatomical location-based classification is categorising the disease into three main types: peripheral neuropathic pain, central neuropathic pain and mixed neuropathic pain (Cheng, 2018). Peripheral neuropathic pain is a type of pain caused due to a lesion or disease that affects the peripheral somatosensory nervous system such as pain caused by ischemic, inflammatory, traumatic, toxic, metabolic, and hereditary. Central neuropathic pain may be caused by stroke , multiple sclerosis, Parkinson's disease, or spinal cord injury. Mixed neuropathic pain can result from both peripheral and central mechanisms such as complex regional pain syndromes, spinal stenosis, lower back pain and cancer pain (Baron, R., 2009). More recently, the idea of an aetiology-based classification of neuropathic pain has been proposed (Gilron, Baron

and Jensen, 2015) which categorizing the disease into five main types: traumatic neuropathic pain, infectious neuropathic pain, metabolic neuropathic pain, toxic neuropathic pain and degenerative neuropathic pain (Gilron, Baron and Jensen, 2015). Figure 1.8 shows a summary of the common classification system of neuropathic pain along with common examples.

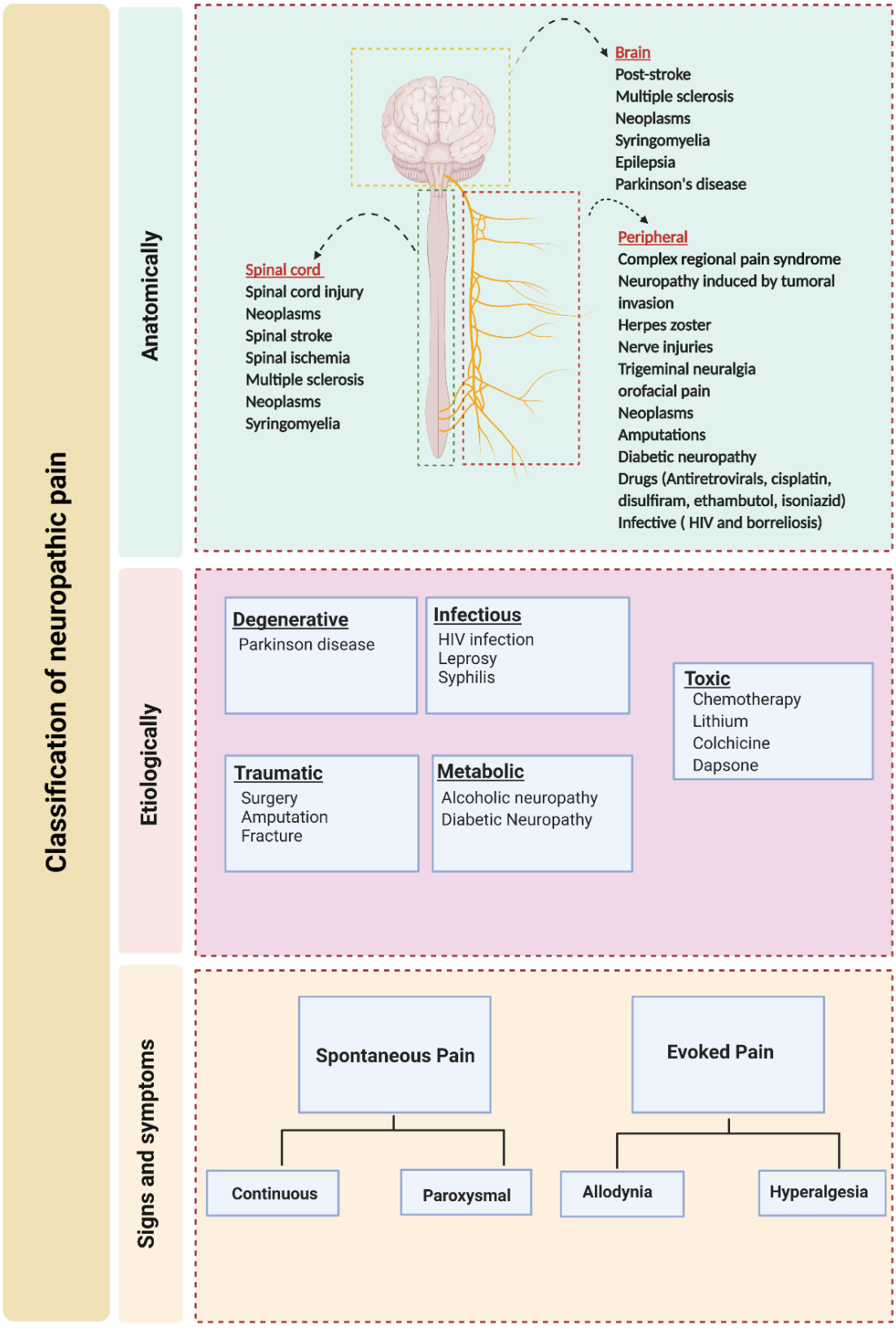


Figure 1.8 A summary of common classification system for neuropathic pain with examples (adapted from Jensen et al., 2001; Baron et al., 2010; Gilron et al., 2015).

1.2.3 Aetiology of neuropathic pain

As previously stated, neuropathic pain is pain initiated or caused by a primary lesion or disease of the somatosensory system. The term 'neuropathic pain' is now widely recognized to encompass a large spectrum of causes and aetiology (Jensen et al., 2001). The aetiology of neuropathic pain is often complex, and for most sufferers, it can be challenging to identify any previous cause that has led to the initiation of the pain symptoms (Satkeviciute, 2018; Jensen et al., 2001). In addition, this type of pain presents in the clinic with different signs and symptoms, which are unique to each person, depending on many factors such as age, sex, and the initial cause of the pain (Woolf and Mannion,1999).

Theoretically, any infection, lesion, or trauma known to create damage or dysfunction to neural tissue (somatosensory pathway) can be considered a potential cause for neuropathic pain (Jay, G.W. and Barkin, R.L., 2014). It can be caused by a multitude of different reasons such as trauma, infection, inflammation, or it can result from many diseases, including; diabetes mellitus, Herpes zoster, human immunodeficiency diseases, multiple sclerosis, trigeminal neuralgia, inflammatory bowel disease, autoimmune diseases, and pancreatitis, neurodegenerative diseases (Stavros, K. and Simpson, D.M., 2014; Freeman, R., 2009; Colloca, L. et al.,2017; Gilron, I et al., 2006). Numerous other causes have also been proposed, including therapeutic interventions such as chemotherapy, radiotherapy, and surgical therapy (Jay, G.W. and Barkin, R.L., 2014 Gilron et al., 2006).

Indeed, several other risk issues are believed to increase the probability of developing neuropathic pain include gender, age, environmental and genetic factors (Colvin and Dougherty, 2015). Gender and age play an essential role since neuropathic pain occurs more in women compared to men (Bouhassira et al., 2008) and the prevalence of neuropathic pain increases with age (Dieleman et al., 2008, Bouhassira et al., 2008). More recently, numerous studies have shown associations between genetic mutation and neuropathic pain (Zorina-Lichtenwalter, Parisien, and Diatchenko, 2018). For example, genetic mutations coding for sodium channel NaV1.6 are linked with

trigeminal neuralgia conditions (Tanaka et al., 2016), mutations in genes coding for μ-opioid receptor 1 are connected with diabetic neuropathic pain (Cheng et al., 2010). In another example, mutations in voltage-gated sodium-channel type IX α subunit (Nav1.7) gene sodium voltage-gated channel alpha subunit 9 (SCN_9A) trigger a spectrum of human genetic pain disorders such as erythromelalgia, which is a very rare condition characterized by neuropathic pain (Drenth and Waxman, 2007; Klein et al., 2013).

1.2.4 Epidemiology of neuropathic pain

It is difficult to estimate the real occurrence and pervasiveness of neuropathic pain (Bouhassira and Attal, 2013). This may be due to many reasons such as multiple etiologies and manifestations (de Paula Posso, de Araújo Palmeira and de Moraes Vieira, 2016), lack of validated diagnostic criteria for all types of neuropathic pain (Chong and Bajwa, 2003), and also the symptoms may be altered significantly from person to person (Colloca *et al.*, 2017). The exact prevalence rate of neuropathic pain within the global population is unknown. However, most of the studies have shown that the best prevalence estimation of neuropathic pain in the general population is likely to lie between 7% and 10% (Yawn et al., 2009; Colloca et al., 2017; Van Hecke et al., 2014; Rosenberger et al., 2020). However, this rate seems to vary across many countries around the world (Van Hecke et al., 2014). Such variation between the countries is not only due to the different regions in which the studies were conducted but also the differences in the methods used for the collection of data, differences in characteristics between individuals (socio-demographics), and also differences in the definitions of neuropathic pain used (Inoue et al., 2017). According to many publications, Canada (17.9%) and United States (9.8% - 12.4%) recorded the highest prevalence rate for neuropathic pain (VanDenKerkhof et al., 2016; Yawn et al., 2009), while Austria (3.3%), Japan (3.2%) and Netherlands(1%) had the lowest prevalence rate (Bouhassira et al., 2008; Gustorff et al., 2008; Harifi et al., 2013). In the UK, France, and Brazil, about 7-10% of chronic pain patients been diagnosed with neuropathic pain (Fayaz et al., 2016).

The role of gender is also an important factor in the chronic pain experience. Chronic pain is more common among females compared to males (Abraham et al., 2018). A study by Bouhassira et al. 2008, has shown that 8% of females suffer from neuropathic pain

compared to 5.7% of males (Bouhassira et al., 2008). The prevalence of chronic pain in particular neuropathic pain increased with age (de Paula Posso, de Araújo Palmeira and de Moraes Vieira, 2016). Indeed, it is essential to know that the prevalence rate of neuropathic pain is increased when associated with a specific condition, for example, among patients with diabetes mellitus this rate increases to 24% in the USA and Turkey (Erbas et al., 2011; Schmader, 2002), 26% in the UK (Lu et al., 2010), 39.6% in Spain (Miralles-García et al., 2010), and 65.3% in Saudi Arabia (Halawa et al., 2010). Furthermore, in patients who have been exposed to the *Herpes zoster* infection, the rate of prevalence increases to 25-50% in patients with lower back pain this increases to 37%- 54.7%; and in patients who have received chemotherapy treatment the prevalence of neuropathic pain is between 25-75% (Veluchamy et al., 2018; Singhmar et al., 2018). The estimated prevalence of chronic pain (chronic low back pain and soft tissue syndromes) with neuropathic characteristics varied from 13% to 43.3% (Fishbain et al., 2014).

A number of recent systematic reviews and meta-analyses have found that chronic alcohol consumers experience a 22-66% increase in neuropathic pain prevalence (Hakim et al., 2019). These values are expected to continue to increase worldwide due to increased prevalence rates of many chronic diseases such as diabetes mellitus and cancer (Cruccu, G. and Truini, A., 2017; Colloca, L., t al.,2017). Table 1.6 shows the prevalence of neuropathic pain in the general population and among individuals with different medical problems.

Table 1.6 Prevalence of neuropathic pain in the general population and in common underlying diseases

	Estimated Prevalence	Reference
General population	7% -10%	(Yawn et al., 2009) (Colloca et al., 2017) (Rosenberger et al., 2020) (Rosenberger et al., 2020)
Peripheral neuropathic pain		
Diabetic neuropathy	14–26%	(Van Acker et al., 2009) (Bouhassira, 2019)
Hypothyroidism	10 -70%	(Penza et al., 2009)
Herpes-Zoster	10–25%	(Bouhassira et al., 2012) (Helgason et al., 2000)
HIV	35%	(Schifitto et al., 2002)
Leprosy	70%	(Ramos et al., 2014)
Cancer	17–39%	(Kerba et al., 2010) (García de Paredes et al., 2011)
Post-surgical	10–50%	(Kehlet et al., 2006) (Guastella et al., 2011)
Post-amputation	60%	(Marchand et al., 2005) (Rosenberger et al., 2020)
Orofacial pain	9 – 26%	(Macfarlane et al., 2009)
Trigeminal neuralgia	99-100%	(Cruccu et al., 2016) (Rosenberger et al., 2020)
Central neuropathic pain		
Multiple sclerosis	20–30%	(Brochet et al., 2009) (Foley et al., 2013) (Rosenberger et al., 2020)
Rheumatoid arthritis	30 -75 %	(Koop et al., 2015)
Guillain-Barre syndrome	55-85%	(Martinez et al., 2010)
Spinal cord injury	53 - 96%	(Siddall et al., 2003) (Hagen and Rekand, 2015)
Stroke	5–30%	(Klit et al., 2011)

1.2.5 Mechanisms and pathophysiology of neuropathic pain

The mechanisms underlying neuropathic pain are not fully understood because of the complexity and possibility that it may triggered by a number of different factors at the same time (Dworkin et al., 2003; Meyer, 2008). However, the majority of research into the field of neuropathic pain has been focused on understanding the molecular mechanisms that underlie this type of pain and the associated changes that occur in both the central and peripheral nervous systems. Animal models of neuropathic pain help researchers to understand the mechanisms that may drive neuropathic pain. These mechanisms include molecular changes in the PNS consists of the afferent nerve fibers and dorsal root ganglion (DRG) and the CNS includes the spinal cord, brainstem, thalamus, and cortex (Ossipov, M.H. and Porreca, F., 2005). Most of the recent theories relate to the mechanisms of neuropathic pain that are, derived from mechanical nerve injury in the animal model, such as spinal nerve ligation (SNL), spared nerve injury (SNI), chronic constriction injury (CCI), and partial scatic ligation (PSL) (Rosenberger *et al.*, 2020; Seltzer, Dubner and Shir, 1990a). Moreover, mechanisms underlying neuropathic pain have been studied in animal models of diabetic neuropathy chemotherapy-induced peripheral neuropathy, Varicella-zoster virus, and human immunodeficiency virus (HIV)–peripheral neuropathy (Rosenberger et al., 2020; Colleoni and Sacerdote, 2010). Additionally, human studies also helped to discover the underlying mechanisms of neuropathic pain such as human surrogate models which based on topical application of high-concentration menthol 40% to healthy volunteers (Magerl and Klein, 2006; Helfert et al., 2013; Vollert et al., 2018). Although the studies in this thesis are focused on the peripheral sensation of neuropathic pain rather than central neural pathways, I will discuss briefly what is known about both peripheral and central neural pathways to provide a more comprehensive picture of how the sensation is provoked and interpreted in the next two sections. Figure 1.9 shows an overview of many of the components involved in peripheral and central mechanisms contributing to neuropathic pain.

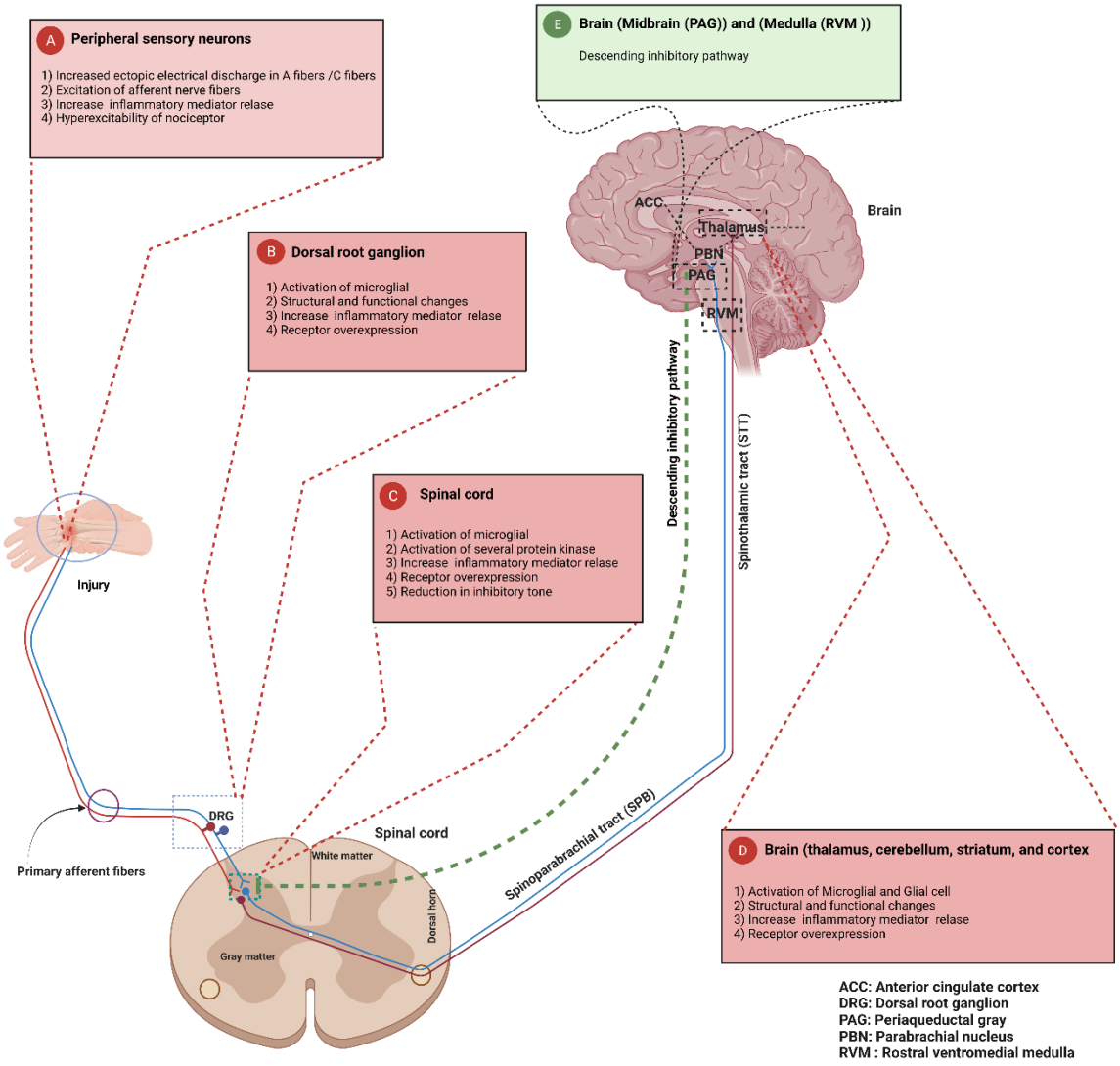


Figure 1.9 Summary of the phenomena which have been observed in the peripheral and central nervous system after peripheral nerve injury, which may contribute to neuropathic pain (adapted from Meacham et al., 2017; Frederick et al., 2017 and Kumar et al., 2018; Osborne et al., 2018).

1.2.5.1 Peripheral mechanisms of neuropathic pain

Several hypotheses have been proposed to explain the peripheral mechanism of neuropathic pain. It is known that the peripheral primary sensory neurons with the cell bodies located in the DRG conduct nociceptive signals, which enters the spinal cord dorsal horn and then is carried to supraspinal structures via ascending pathways (Kocot-Kępska et al., 2021). The peripheral somatosensory nervous system can be damaged in a variety of ways such as mechanical, thermal, chemical, and infectious. Multiple preclinical and clinical studies demonstrated that the peripheral nerve endings of pain-processing (C and A fibres) damaged by toxins, cytokines, and inflammatory mediators

(Bouhassira et al., 2005; Haroutounian et al., 2014; Oliveiraet al., 2016; Kocot-Kępska et al., 2021). It has been observed that nociceptive neurons (A and C), as well as peripheral nerve endings along the axon and DRG neurons, experience extensive functional, structural, and molecular changes following a peripheral nerve injury in animal models of neuropathic pain (Kuner and Flor, 2017; Kocot-Kępska et al., 2021). After peripheral nerve injury, inflammatory mediators, and neurotransmitters such as chemokines and leukocytes, noradrenaline, bradykinin, histamine, prostaglandins, cytokines, serotonin, and neuropeptides are released at the site of injury (Oliveiraet al., 2016). This release leads to changes in the properties of primary afferent neurons (Scholz and Woolf, 2007; Chen et al., 1999; Romero-Sandoval et al., 2008). These changes in the expression of neurotransmitters and neuropeptides lead to the development of mechanical and thermal hypersensitivity and act to sensitise nociceptors to further neural input (Ossipov and Porreca, 2005). Also, these changes alter the number and expression of ion channels, such as Na^+ and Ca^{2+} channels across the membrane in injured nociceptor nerve fibres and their dorsal root ganglia, which causes the spontaneous generation of action potentials, leading to neuronal hyperexcitability and ultimately an increase in ectopic firing (Bridges, D. et al.,2001; Beydoun, A. and Backonja, M., 2003). This ultimately causes an increase in the response of the nociceptors to thermal and mechanical stimuli. This mechanism is known as peripheral sensitization (Siddall, P.J. and Cousins, M.J., 1997).

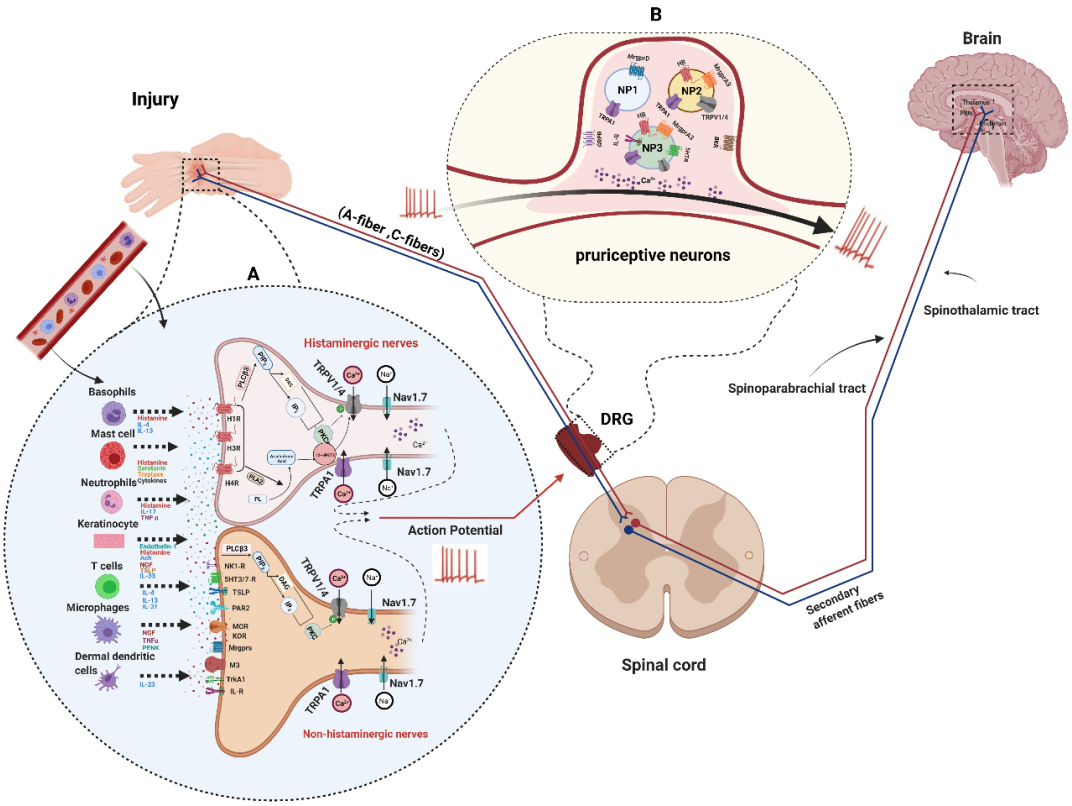
Another hypothesis that explains the peripheral mechanism of neuropathic pain is the ephaptic nerve coupling hypothesis (Blumberg and Jänig, 1982). Under normal conditions, each of the nerve fibres is isolated from each other (Amir, R. and Devor, M., 1996). However, under pathophysiological conditions or peripheral nerve injury, an electrical connection between injured nerve fibres and nearby uninjured fibres takes place (cross excitation) (Blumberg, H. and Jänig, W., 1982; Bridges, D. et al., 2001). This type of transmission might be responsible for the peripheral abnormal pain sensation that follows peripheral nerve lesions (Blumberg, H. and Jänig, W., 1982).

Symptoms that are present in patients with neuropathic pain include allodynia, hyperalgesia, dysesthesias and paresthesias. These can manifest singularly, or patients can experience more than one symptom (Jensen, T.S. and Finnerup, N.B., 2014;

Finnerup, N.B. et al., 2001). Several lines of evidence suggest that these symptoms are due to functional and biochemical changes of peripheral sensory fibres (myelinated Aβ, Aδ, and unmyelinated C fibres). These changes sometimes appear as spontaneous pain in the clinical setting (Siddall, P.J. and Cousins, M.J., 1997). Earlier research has shown that hyperalgesia may arise from continuous discharge in C-fibres primary afferent neurons (Byers, M.R. and Bonica, J.J., 2001), whereas dysesthesias and paresthesias may be caused by intermittent spontaneous discharges in A-β or A-δ fibres (Rowbotham, M.C. and Petersen, K.L., 2001).

Additionally, damaged primary sensory neurons and loss of function are some of the hypotheses used to explain the peripheral mechanism of neuropathic pain. After peripheral nerve injury, neurodegeneration interrupts the electrical connection between the peripheral and central nervous system, which subsequently leads to sensory loss (Rosenberger et al., 2020). Axonal transection occurs at sites of injury of primary sensory neurons destroy the distal axons due to a degenerative process called Wallerian degeneration (Colleoni and Sacerdote, 2010; Campbell and Meyer, 2006). This process affects primary sensory neurons at the site of injury, in particular the small-fiber neurons containing nociceptors (Tandrup et al., 2000). After that, abnormal afferent input may stimulate the degeneration of superficial dorsal horn neurons mediated by glutamate excitotoxicity (Scholz et al., 2005).

Another peripheral mechanism that is undergoing extensive studies is the coupling between the sympathetic nervous system and the sensory nervous system (Attal, N., 2000; Bridges, D. et al., 2001). After an injury in the PNS, an abnormal interaction between the sympathetic and the sensory nervous system takes place, which leads to an increase in the sensitivity to catecholamines in a small subset of patients, that result in the development of neuropathic pain (Bridges, D. et al., 2001; Pasero, C., 2004). Moreover, neuropathic pain is frequently referred to as `sympathetically maintained pain' in some patients (Attal, N., 2000; Pasero, C., 2004). This mechanism is not completely clear, and it needs future investigation that will allow many questions to be answered in terms of how and where the sympathetic nervous system is coupled with the sensory nervous system to produce neuropathic pain. Figure 1.10 shows peripheral mediators and their receptors involved in neuropathic pain pathway.



ACC: Anterior cingulate cortex
Bhlhb5: Helix-loop-helix family member B5
BK: Bradykinin
B1/2-R: Bradykinin receptors 1 and 2
Dyn:dynorphin
Gly: Gycine
Glu: gutamate
GRP: Gastrin-releasing peptide
GRPR: Gastrin-releasing peptide receptor
GABA: Gamma-amino butyric acid
His: Histamine
H1R:Histamine 1 receptor
H4R: Histamine 4 receptor
LCN2 :lipocalin-2

IFN-α: Interferons
Mor: Morphine
MOR:μ-opioid receptors
P:Protease
PAG: Periaqueductal gray
PAR2:Protease-activated receptor-2
PMA: premotor area
KOR:κ-opioid receptors
SP: Substance P
SI: Primary somatosensory cortices
SII: Secondary somatosensory cortices
SMA: Supplementary motor area
TRPA: Transient receptor potential ankyrin
TRPV: Transient receptor potential vanilloid
5-HT:Serotonin
5-HT3/7-R: Serotonin receptors type 3 and 7

Figure 1.10 Diagram showing peripheral mediators involved in neuropathic pain pathway. (adapted from Gilron et al., 2015; Oliveiraet al., 2016; Dureja et al., 2017; Yam et al., 2018; Rosenberger et al., 2020)

1.2.5.2 Central mechanisms of neuropathic pain

Central mechanisms of neuropathic pain play a significant role in the pathophysiology of this type of pain. A key mechanism for identifying neuropathic pain is defined as central sensitisation, which is explained by the IASP as "*increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input*" (Loeser and Treede, 2008). After spinal cord injury, there is an increased

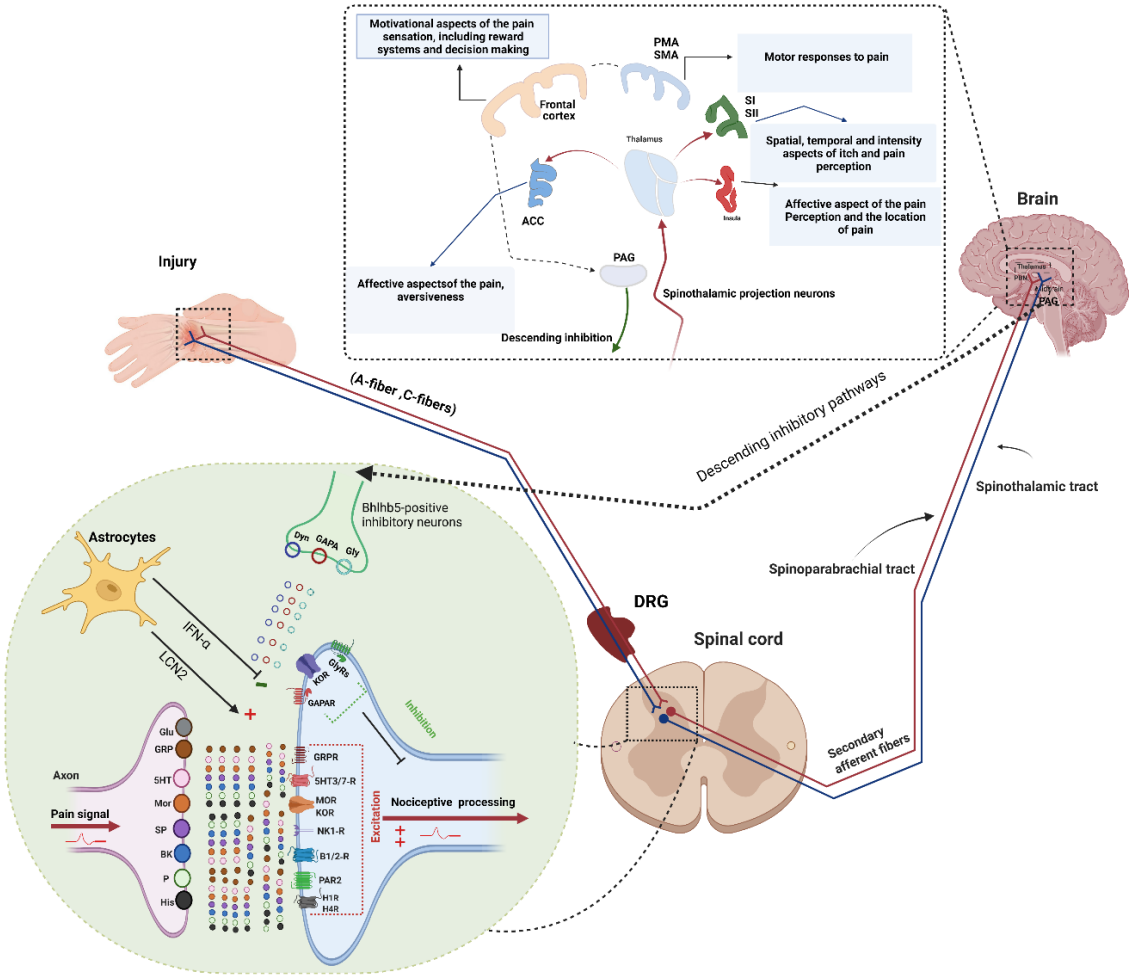
production of neurotransmitters (e.g., glutamate, calcitonin gene-related peptide, and tachykinins (e.g., neuropeptides substance P and neurokinin A) in the spinal cord (Beydoun and Backonja, 2003; Cohen and Mao, 2014). Continued release of these mediators leads to increased intrinsic excitability of second-order neurons in the spinal cord level (Costigan et al., 2009) and activation of the N-methyl-D-aspartate (NMDA) receptor, which leads to an increase in intracellular Ca^{2+} levels (Siddall and Cousins, 1997). An increase in the intracellular Ca^{2+} leads to many changes at the spinal cord level, including the stimulation of protein kinase C (PKC), phospholipase C (PLC), nitric oxide synthetase, mitogen-activated protein kinases (MAPK), Src-kinases family (SFKs), extracellular signal-regulated kinase (ERK), calcium/calmodulin-dependent kinase II (CaMKII) and altered the gene expression of; all of these changes lead to the development of central sensitization. The net effect of these changes that is the excitability of the C-fibre and activation of the NMDA receptor is the'' wind-up'' phenomenon which contributes to the development of hyperalgesia (Bridges et al., 2001).

Central disinhibition is a mechanism that contributes to the growth and maintenance of neuropathic pain. This phenomenon occurs when the production of inhibitory neurotransmitters in the spinal cord and brainstem neurons decreases or is suppressed. This leads to a state of disinhibition that is associated with loss of inhibition (Yaksh, T. L. 1989; Meacham, K. et al., 2017). In neuropathic pain states, disinhibition in the superficial dorsal horn is associated with decreased levels of GABA and its receptors. The loss of glycine inhibitory currents could be attributed in part to apoptosis of inhibitory neurons, (Moore et al., 2002; Scholz et al., 2005). This neuronal death upon injury might result in excitotoxicity developed by NMDA receptors for a long time instead of the large amounts of glutamate distribution centrally (Scholz et al., 2005). Indeed, according to brain imaging studies and electrophysiological investigations in patients with neuropathic pain indication that neurodegeneration may also occur in some area of CNS (Costigan, Scholz and Woolf, 2009). Figure 1.11 shows central mediators and their receptors involved in neuropathic pain pathway.

It is also important to mention the descending inhibitory pathway that occurs after pain activation. Dorsal horn neurones at spinal cord level receive efferent inputs from

descending fibres, the effects of this efferent inputs may be direct or indirect *via* involvement of inhibitory interneurons which contain GABA and Glycine (Barber et al., 1982; Todd and Sullivan, 1990). Endogenous inhibitory pathway that is involved in the modulation and control of pain was initially proposed by Melzack and Wall in 1965 (Melzack and Wall, 1965). Melzack and Wall proposed the gate control theory of pain where brain impulses can influence spinal nociceptive transmission *via* cutaneous stimulation of large afferent fibres (Aβ-fibres); this can selectively inhibit C-fibres (Besson and Chaouch, 1987; Woolf and Wall, 1982). Such an inhibitory influence can be exerted on both nocispecific and multireceptive neurones in the dorsal horn of the spinal cord either directly by activation of interneurones (GABA and glycine) (Todd, 1996; Hughes and Todd, 2020) or indirectly by activation of descending pathways from the supraspinal areas. In addition to this, monoamines (such as serotonin and noradrenaline) can be directly released by descending control systems to exert inhibitory effects (Besson and Chaouch,1987).

Overall, the pathophysiology of neuropathic pain is quite complicated and despite years of continuous research, most of the research is obliged to explore various pathways and one of these pathways ~~is~~ contributes to the histaminergic system and hyperalgesia which are the major topics of this thesis.



ACC: Anterior cingulate cortex
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GABA: Gamma-amino butyric acid
His: Histamine
H1R:Histamine 1 receptor
H4R: Histamine 4 receptor
LCN2 :lipocalin-2

IFN-α: Interferons
Mor: Morphine
MOR:μ-opioid receptors
P:Protease
PAG: Periaqueductal gray
PAR2:Protease-activated receptor-2
PMA: premotor area
KOR:κ-opioid receptors
SP: Substance P
SI: Primary somatosensory cortices
SII: Secondary somatosensory cortices
SMA: Supplementary motor area
5-HT:Serotonin
5-HT3/7-R: Serotonin receptors type 3 and 7

Figure 1.11 Diagram showing spinal receptors involved in neurophathic pain pathway. (adapted from Gilron et al., 2015; Dureja et al., 2017; Rosenberger et al., 2020; Finnerup et al., 2021)

1.2.6 Treatment options for neuropathic pain

The management of neuropathic pain is still one of the major challenges facing healthcare organizations. Reasons for this difficulty in the management of this type of chronic pain include many factors, such as a lack of validated diagnostic criteria for all types of neuropathic pain (Chong and Bajwa, 2003) and lack of a consistent and simple clinical instrument that can identify the characteristics of neuropathic pain (Bouhassira et al., 2008). Also, other complex factors include the fact that neuropathic pain involves several pathophysiological mechanisms that cannot be controlled by a single treatment option (Bouhassira and Attal, 2011). Other management difficulties include a lack of awareness among the patients regarding the importance of early diagnosis and treatment of neuropathic pain. Furthermore, poor patients compliance due to unwanted adverse effects of the pain medications and the need for multiple daily dosing adds to the challenges associated with the management of neurophathic pain (Tölle, 2010). In patients with refractory neuropathic pain, combination therapy involving two drugs with synergistic mechanisms of action may be more effective than single agents alone. However, this strategy may lead to increased risk of drug-drug interactions and subsequently increased risk of adverse drug events (Haanpää et al., 2010). All these factors make the treatment of neurophathic pain more difficult.

Generally, the management of neuropathic pain focuses on a control of the symptoms and aetiological conditions such as diabetes or infections (Colloca et al., 2017). Patients with neuropathic pain do not respond to most analgesics medications such as paracetamol, nonsteroidal anti-inflammatory drugs (NSAIDs), or opioids (Brooks and Kessler, 2017). The available neuropathic pain treatments include different strategies and protocols that contain pharmacological, non-pharmacological, and interventional therapies (Brooks and Kessler, 2017; Gilron, Baron and Jensen, 2015).

Pharmacological management of chronic neuropathic pain continues to be the most effective treatment option, but results are still insufficient, and only a small percentage of patients experience adequate pain relief (Finnerup, Sindrup and Jensen, 2010). Therefore, the IASP and other pain authorities have introduced treatment guidelines

that sort the most commonly used drugs for alleviating neuropathic pain symptoms into varying levels according to efficacy, safety, tolerability, drug interactions, side effects and their impact on health-related quality of life (Freynhagen and Bennett 2009). These varying levels allow consistent first-line and second-line treatment recommendations. According to these authorities, the first-line of treatment for neuropathic pain includes the use of calcium-channel blockers, i.e. (gabapentin and pregabalin), tricyclic antidepressants, and serotonin reuptake inhibitors, i.e. (nortriptyline, desipramine, duloxetine, and venlafaxine) as well as topical lidocaine with low recommendation (Gilron, Baron and Jensen, 2015). Opioid analgesics and tramadol are recommended as a second-line treatment option (Moulin et al., 2007; Gilron, Baron, and Jensen, 2015). Other antiepileptic and antidepressant medication, such as carbamazepine, lamotrigine, oxcarbazepine, valproic acids, bupropion, topiramate, paroxetine, and other adjunctive drugs like clonidine, capsaicin, dextromethorphan, memantine and mexiletine, would generally be used as third-line treatment options (Freynhagen and Bennett 2009; Dworkin et al., 2007; Gilron, I, et al., 2006). However, patients who have not responded to monotherapy or have developed side effects with high doses of monotherapy might need a combination of two or more of other recommended medications from the alternative options (Dworkin et al., 2010; Finnerup, et al.,2010). Figure 1.12 represents an algorithm for the pharmacologic therapy of neuropathic pain.

Additionally, non-pharmacological therapies such as stress management, good sleep hygiene, physical therapy and other potentially valuable interventions might help patients who have not responded adequately to medication management or those who have developed severe side effects (Dworkin et al., 2010). It is essential to know that some patients with neuropathic pain do not respond to pharmacological and non-pharmacological treatment either alone or in combination (Hansson et al., 2009; Gilron, Baron, and Jensen, 2015). Therefore, according to many clinical studies the interventional strategies should be considered in order to control this type of refractory pain (Gilron, Baron and Jensen, 2015; Dworkin et al., 2013). Interventional strategies including nerve blocks, neuromodulation, spinal cord stimulation, and neurosurgical interventions seem to be effective in treating and helping to control many types of peripheral and central neuropathic pain conditions such as postherpetic neuralgia,

diabetic neuropathy, spinal cord injury, central poststroke pain, trigeminal neuralgia and trigeminal neuropathy (Dworkin et al., 2013).

Overall, neuropathic pain frequently does not respond well to standard and existing pain therapies and may even deteriorate rather than improve over time. Thus, it can result in severe disability in specific individuals. However, efforts have been undertaken in recent years to improve our understanding of the neuropathic pain mechanism. This effort opened up novel options to find more effective treatments for neuropathic pain. An example, may include targetting peripheral histaminegic system as a potential novel therapeutic target for many pathological conditions, including neuropathic pain. These may incluse targetting either hisatmine receptors or histamine alone and this approach will be explored in this present work to provide new therapeutic options for patients suffering from neuropathic pain.

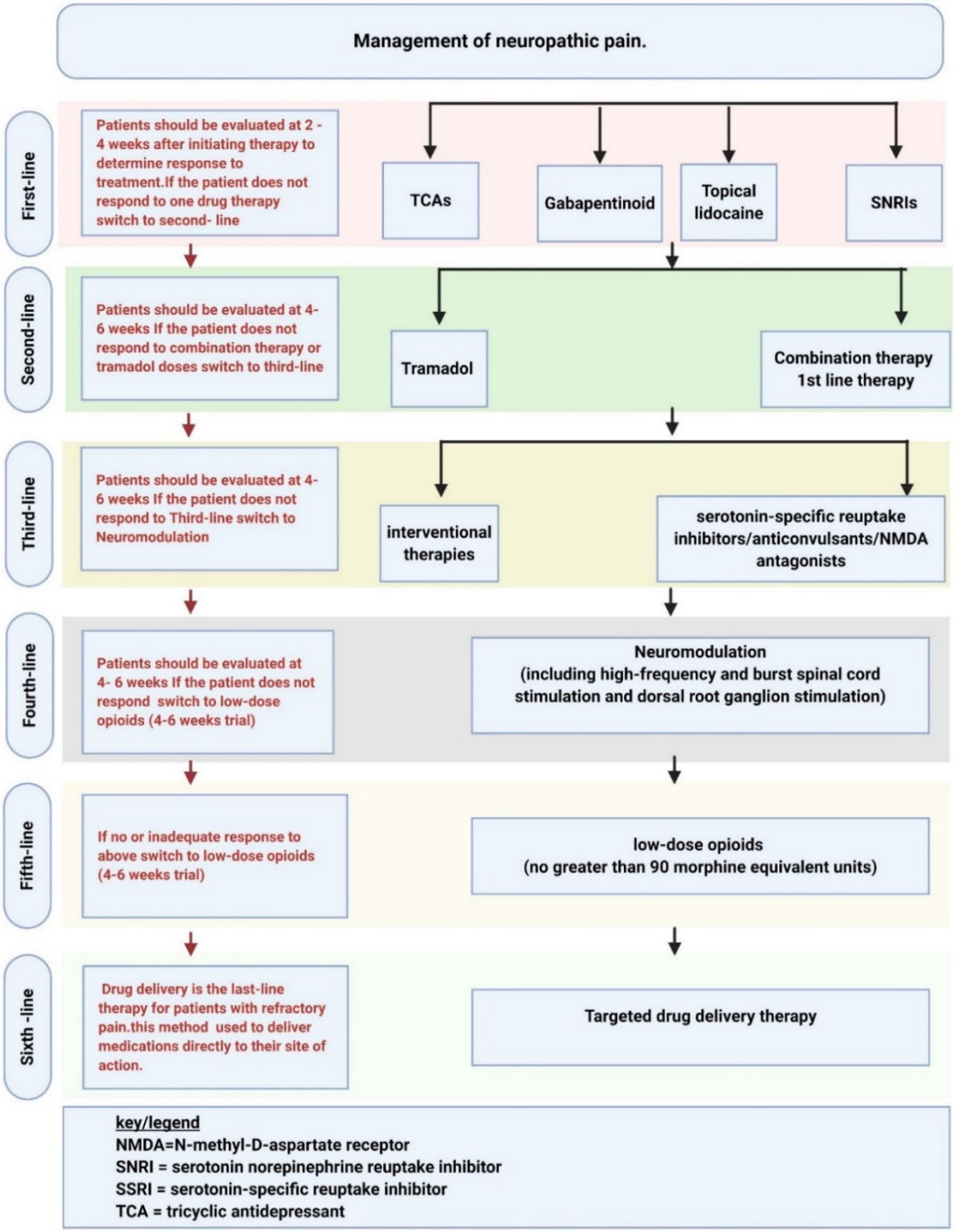


Figure 1.12 Comprehensive algorithm for the management of neuropathic pain. (adapted from Bates et al., 2019; Fornasari, 2017; Chong and Bajwa, 2003; Jensen et al., 2001)

1.2.7 Animal models of neuropathic pain

Animal models of neuropathic pain are crucial for understanding the underlying mechanisms of this disease, and they are important for the development of effective treatment options (Rice et al., 2018). Without these models, it would be challenging to study and understand the pathophysiology of pain at the molecular level. Over the past two decades, numerous animal models of neuropathic pain have been developed and characterized to simulate the clinical future of pain with miscellaneous aetiology (Jaggi et al., 2011). Indeed, these models are highly reproducible and continuously elicit allodynia and hyperalgesia behavioral symptoms in response to thermal and mechanical stimulation (Niederberger and Geisslinger, 2008). There is an abundance of published studies describes different animal models to assess neuropathic pain pathophysiology (Table1.7). Some of these models are used to study peripheral neuropathic pain mechanisms, whereas others to study central mechanisms (Jaggi et al., 2011). During the last three decades, several models for peripheral neuropathic pain have been developed and at present they are used in laboratories around the world. The majority of these models share alterations in hind-limb cutaneous sensory threshold following a partial injury of a peripheral nerve (usually sciatic) as a common feature (Wall et al.,1979). The four most commonly used models are: chronic constriction injury (CCI), partial sciatic nerve ligation (PSL), spinal nerve ligation (SNL) and spared nerve injury (SNI) (Bennett & Xie, 1988; Seltzer et al., 1990; Kim, S. H., and Chung, J. M. 1992; Decosterd & Woolf, 2000). The neighboring nerve fibres, which are next to the injured ones, are believed to play a critical role in developing neuropathic pain symptoms such as hyperalgesia and allodynia (Ma et al., 2003; Wu et al., 2001). In addition, prior research has established that central neuropathic pain could be induced after injury to the dorsal root ganglion (DRG) (Hu and Xing, 1998), dorsal root (Eschenfelder et al., 2000) or even the ventral root (Sheth et al., 2002). Figure 1.13 shows a summary diagram for the most common animal models of both central and peripheral neuropathic pain.

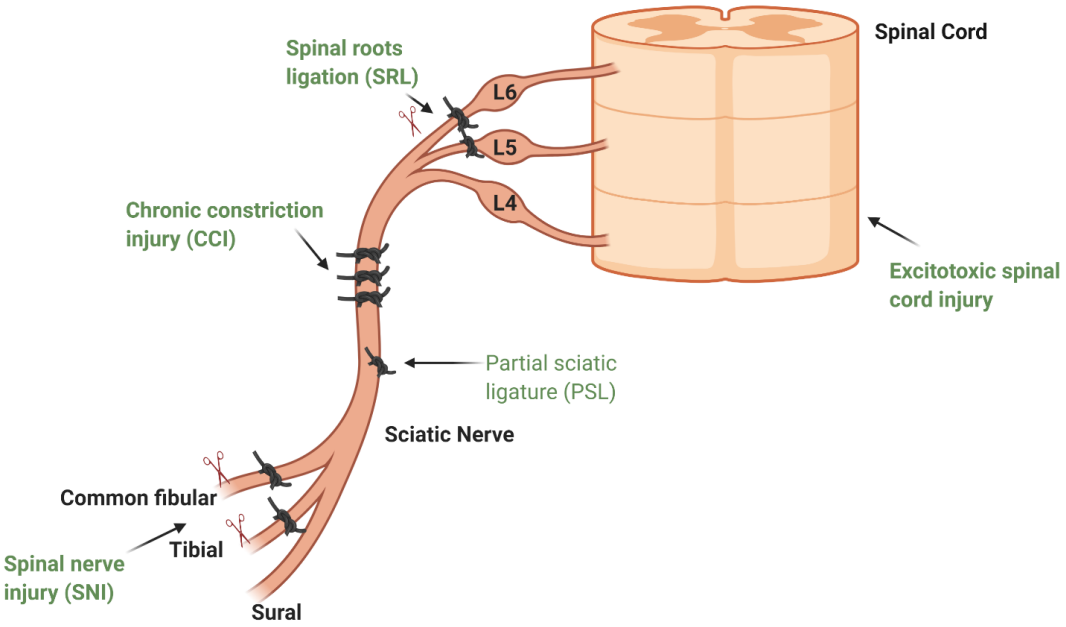


Figure 1.13 A summary diagram for the most common animal models of neuropathic pain. The sciatic nerve innervates the hind paw and the cell bodies of the sciatic nerve lie within the dorsal root ganglia, located in L4, L5 and L6. Partial nerve ligation (PNL), chronic constriction injury (CCI), spinal roots ligation (SRL) and spinal nerve injury (SNI). All of these nerve injuries cause damage to only a portion of the afferents from the foot, sparing some axons and allowing for the measurement of pain hypersensitivity *via* reflexive hindpaw withdrawal responses. (adapted from Martin et al., 2003; Ma and Zhang, 2011; Austin and Moalem-Taylor, 2013; Sousa et al., 2016).

Table 1.7 Most common central and peripheral neuropathic pain models

Model name	Type of injury	Animal	Reference
Central neuropathic pain models			
Spinal cord injury by contusion	A weight is dropped on exposed spinal cord	Rats and mice	(Kang *et al.*, 2006)
Excitotoxic spinal cord injury	Spinal injection of aminoacids	Rats and mice	(Gorman *et al.*, 2001)
Spinal hemisection	Laminectomy of T11-T12	Rats	(Christensen et al., 1996)
Photochemically induced injury	Spinal cord injury initiated photochemically.	Rats	(Watson *et al.*, 1986)
Peripheral neuropathic pain models			
Axotomy	Complete sciatic section	Rats	(Kim, Yoon and Chung, 1997)
Chronic sciatic constriction	Four ligatures around the sciatic nerve (loose) Three ligatures around the sciatic nerve (loose)	Rats and mice	(Bennett and Xie, 1988)
	Ligature of 1/3 to ½ the nerve (tight)	Rats and mice	(Malmberg and Basbaum, 1998) (Seltzer, Dubner and Shir, 1990b)
Spinal roots ligation	Ligation of L5/L6 (tight) Ligation of L7 (tight)	Rats and monkeys	(Carlton *et al.*, 1994) (Kim and Chung, 1992)
Spared Nerve injury	Tibial and peroneal axotomy	Rats and mice	(Decosterd and Woolf, 2000)
Common peroneal ligation	Common peroneal ligation	Mice	(Vadakkan, Jia and Zhuo, 2005)
Sciatic cryoneurolysis	Freezing the proximal sciatic nerve	Rats	(DeLeo *et al.*, 1994)
Balloon-induced sciatic injury	Implant of polyethylene balloon around the nerve	Rats and mice	(Vanický *et al.*, 2001)
Laser-induced sciatic injury	Decreased blood flow to the nerve mediated by radiation	Rats	(Kupers *et al.*, 1998)
Diabetes-induced neuropathy	Persistent changes in nerves induced by hyperglycemia	Rats and mice	(Courteix *et al.*, 1994)
Alcoholic neuropathy	Ethanol administration for long periods	Rats	(Bosch *et al.*, 1979)
Pyridoxine-induced neuropathy	Administration of high doses of pyridoxine for long periods	Dogs and rats	(Chung *et al.*, 2008) (Callizot, Warter and Poindron, 2001)
Trigeminal neuralgia	Trigeminal compression; chronic infraorbitary nerve constriction	Rats	(Dao-Shu Luo *et al.*, 2012)
Orofacial pain	Formalin or complete Freund's adjuvant injection in temporomandibular joint and jaw	Rats and mice	(Clavelou *et al.*, 1995) (Ren, 1999)

1.3 Histamine and its receptors

Histamine is arguably one of the most important neurotransmitters and neuromodulator in the mammalian body (Haas et al., 2008; Obara et al., 2020). It regulates several physiological functions, such as biological rhythms, energy metabolism, sleep and waking cycle, body weight, food intake, body temperature and behavioral state (Haas et al., 2008; Lindskog, 2017). In addition, histamine is a key mediator in many pathological conditions, including immune pathology (Jutel et al., 2009), neurological and psychiatric disorders insomnia (Krystal et al., 2013) chronic neuropathic pain (Obara et al., 2020), inflammation (MacGlashan, 2003), allergy (Thurmond et al., 2008), motion sickness (Takeda et al., 1986) and itch (Baron et al., 2001). The studies in this thesis are focused on the role of peripheral histaminergic system in moduation of acute itch and neurophathic pain. Therfore, I will discuss briefly what is known about hisatmine history, releases, receptors, signaling pathway and its role in acute itch and chronic pain in the following section.

1.3.1 History and background

Histamine (2-(4-imidazolyl)-ethylamine) is one of the most intensely studied amino acid-derived immunomodulator molecules. It was discovered by the British physiologist Sir Henry Hallett Dale in 1910 (Dale and Laidlaw, 1910). Eight years later, in 1918 Dale called this compound 'histamine' , which comes from the Greek word for tissue *histos* (Dale and Richards, 1918). In 1932, histamine was identified as a mediator of anaphylactic reactions (Burns *et al.*, 2008). By 1937, Bovet and Staub, synthesised the first antihistamine phenolic ether thymoxyethyl-diethylamine (Staub and Bovet, 1937). However, due to severe toxic effects of this compound it was not used clinically. Despite the unsuccessful story of the first antihistamine, it opened a new door *via* targeting histamine system to control pathological conditions. After five years, in 1942, Halpem synthesised the first clinically used antihistamine – phenbenzamine for the symptomatic relief of allergic (Halpern, 1942). Subsequently, in 1944 mepyramine (also known as pyrilamine) was released in France for treatment of allergic conditions (Bovet, Horclois and Fournel, 1944). In 1966, Ash and Schild from the Department of Pharmacology, University of London discovered the first histamine receptor and suggested the symbol

H_1 and the they called drugs which antagonize the H_1R as antihistamine dugs (Ash and Schild, 1966). The discovery of selective ligands for H_1R played an importanat role in understanding ot the pharmacological properties and effects of H_1R. Later in 1972, Black et al., defined the H_2R and its role in the gastric acid secretion in the stomach (Black *et al.*, 1972). Then, in 1977, Brimblecombe et al., develpoed the first clinically used H_2R antagonist (cimetidine) that inhibited gastric acid secretion (Brimblecombe *et al.*, 1975). Bradshaw et al., also discovered more potent H_2R antagonist (ranitidine), which had a lower side effect and less drug-drug interaction with improved pharmacokinetic properties comparing to cimetidine (Bradshaw *et al.*, 1979).

In 1983, Arrang et al., noted that many histamine receptor ligands modulated histamine actions in the central nervious system but the effect did not match H_1R or H_2R, and from this point they recognised H_3R (Arrang et al., 1983). Fifteen years later, in 1999, a group of scientists discovered a novel H_4R by using genmic- based approaches (Lovenberg *et al.*, 1999). In 2003 and 2004, first highly selective H_4R antagonist JNJ 7777120 was developed (Jablonowski *et al.*, 2003; Thurmond *et al.*, 2004). Finally, in 2017, Kolib-Sielecka et al., developed the first H_3R invasive agonist used in the clinical filed for the treatment narcolepsy with or without cataplexy in adults narcolepsy (Kollb-Sielecka *et al.*, 2017; Tiligada and Ennis, 2020). Figure 1.14 displays the greatest achievements in histamine research that have occurred since Sir Henry Hallett Dale critical discovery.

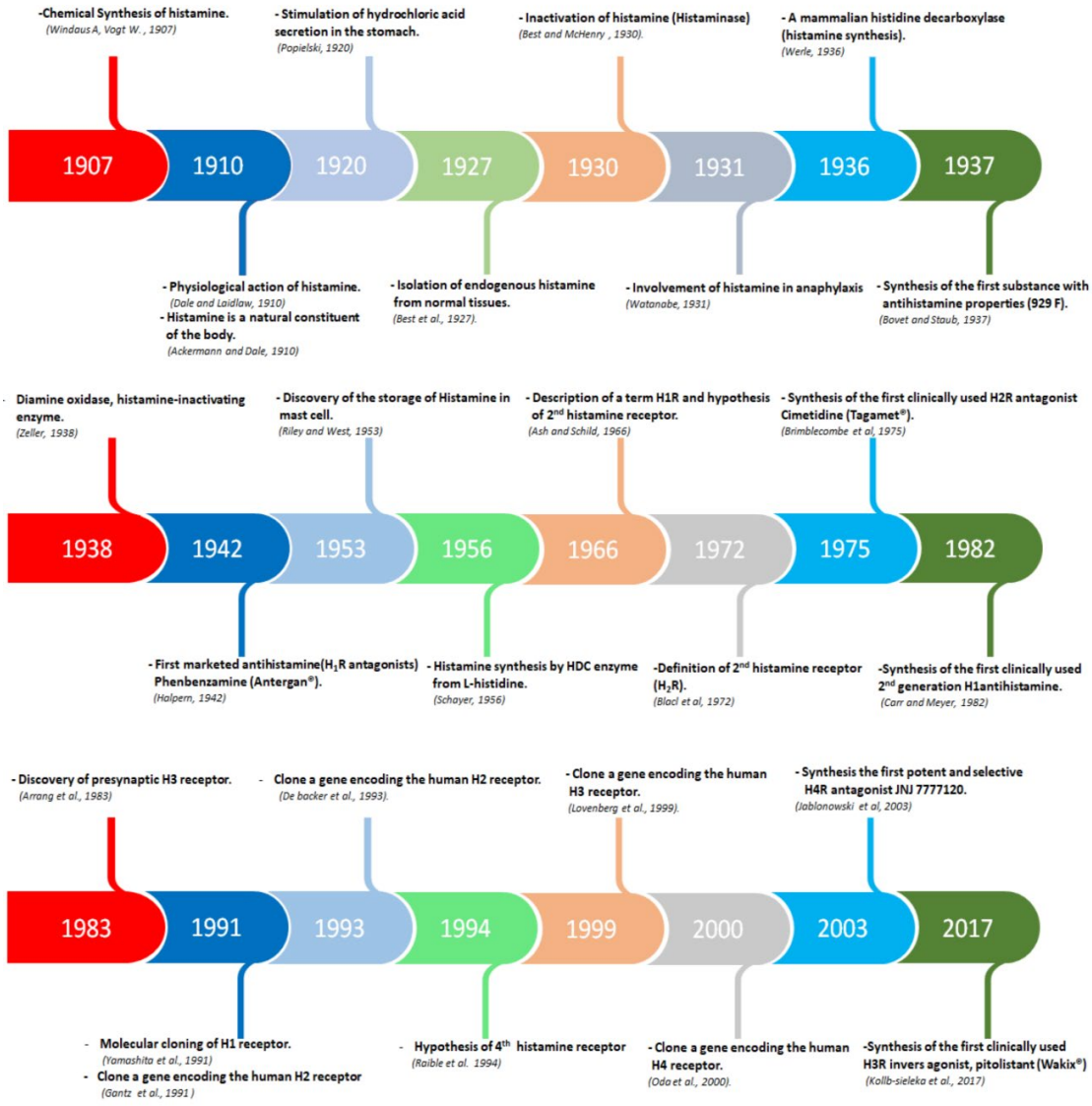


Figure 1.14 Timeline of the significant accomplishments in histamine research (adapted from Cataldi et al., 2014 and Tiligada and Ennis, 2020)

Histamine is found in almost all tissues of the human body (Haas HL, 2008) and it is synthesised and stored primarily in cytosolic granules of the peripheral and central tissues, mast cell, basophils, eosinophils, platelets, basophiles, histaminergic neurons and enterochromaffine cells (Branco *et al.*, 2018; Benly, 2015). Histamine is produced from the amino acid L-histidine *via* oxidative decarboxylation reaction under the effect of histidine decarboxylase enzyme (HDC) (Bodmer, 1999; Haas, H.L. et al, 2008). After synthesis of histamine, these cells store histamine in cytosolic granules (Branco *et al.*, 2018; Benly, 2015; Oguri and Yoneya, 2002). However, numerous types of cells such as epithelial cells dendritic cells, neutrophils and T lymphocytes do not store histamine but

synthesize and secrete it immediately after production (O'Mahony, Akdis and Akdis, 2011). Histamine action is terminated by metabolic breakdown either by oxidative deamination *via* diamine oxidase (DAO) or by methylation *via* histamine-N-methyltransferase (NMT) (Schwelberger *et al.*, 2013). Figure 1.15 demonstrates the chemical reaction that catalyzes the formation of histamine from histidine using the enzyme histidine decarboxylase (HDC).

Histamine is produce in many mammalian tissues, including skin, lung, abdominal mucosa, heart, lung, blood vessels and nerve endings in CNS and PNS (Carraway *et al.*, 1982). It is normally stored in mast cells, enterochromaffin cells, basophils and specific type of neurons (Haas, Sergeeva and Selbach, 2008). Under normal conditions, histamine stored in secretory granules inside the cells with heparin and chymotrypsin, a chemotactic factor (Anderson and Uvnäs, 1975; Lagunoff, Martin and Read, 1983). Under pathological condition such as nerve damage, toxin or disease-related reaction or in response to different chemical, mechanical or thermal stimuli mast cell degranulated and release active mediators, primarily histamine (Carraway et al., 1982; Kaur et al., 2017).

Histamine exists in both the PNS and CNS is involved in numerous and multiple physiological and pathological processes (Hill *et al.*, 1997; Parsons and Ganellin, 2006). The physiological functions include vasodilatation, stimulates heart rate and increase cardiac inotropy, contraction of smooth muscles in different organ such as airways, uterus, stomach and intestine, cell proliferation, sleep-waking cycle, neurotransmission, immunomodulation, hematopoiesis, synaptic plasticity, hormonal secretion, thermoregulation, wound healing, cognition, learning and memory formation (Maintz and Novak, 2007; Parsons and Ganellin, 2006; Panula *et al.*, 2015). On the other hand, histamine is involved in multiple pathological condition such as headache, multiple sclerosis, inflammation, and allergy, pruritus, narcolepsy, cognitive disorders, Alzheimer's disease, obesity, attention impairment, and neuropathic pain as well as stimulates the cancer cells proliferation and promotion (Hegyesi *et al.*, 2005; Maintz and Novak, 2007; Smuda and Bryce, 2011) (Yu, J., et al.,2013).

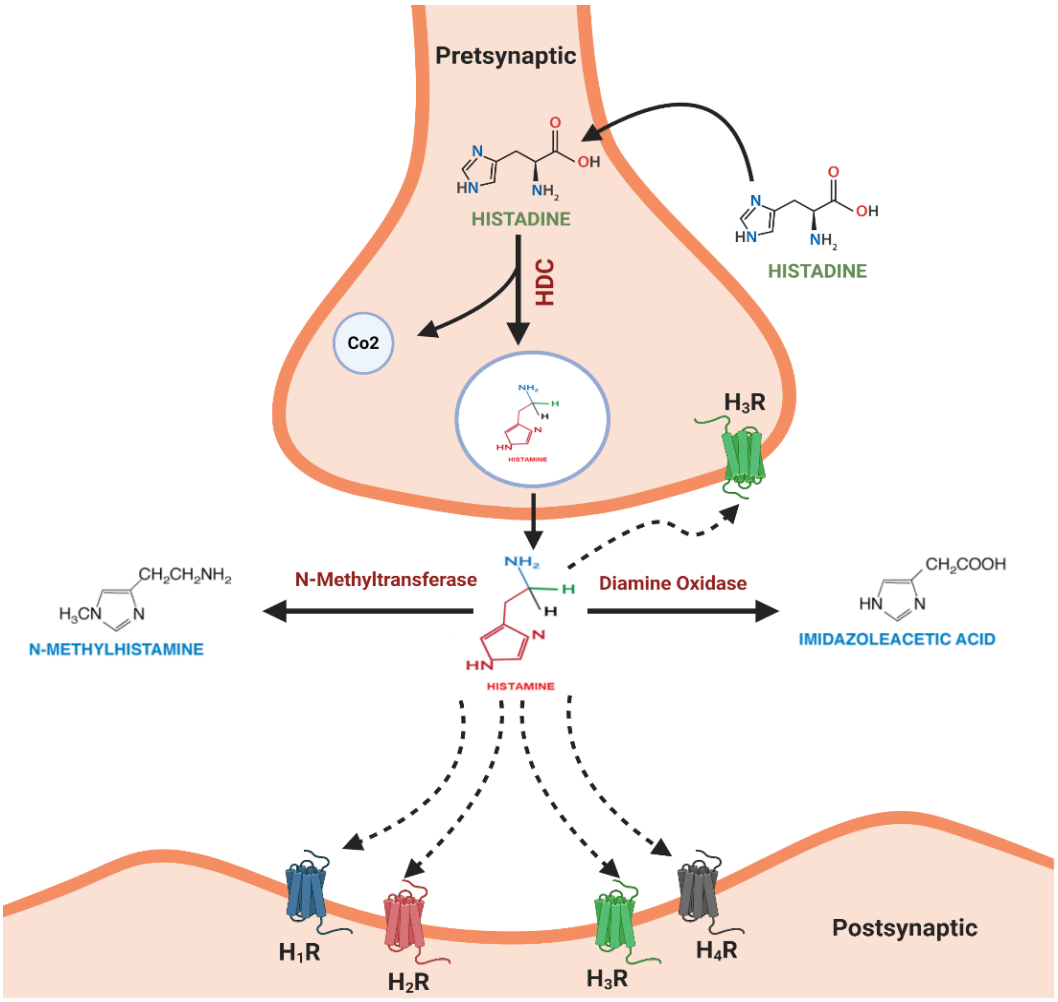


Figure 1.15 Chemical structures and synthesis and inactivation of histamine. Histamine can be released from a variety of cells including neurons, enterochromaffin-like cells, and mast cells. Histamine is generated by decarboxylation of histidine by the enzyme histidine decarboxylase (HDC). It can be inactivated either by methylation by histamine N-methyltransferase (HMT), or by oxidative deamination by diamine oxidase (DAO). (adapted from Haas et al., 2008; Huang et al., 2018).

1.3.2 Distribution and function of the histamine receptors

Histamine exerts its effects *via* a family of receptors, known as the histamine receptors. Currently, there are four known histamine receptors: H_1R, H_2R, H_3R, and H_4R, which are G protein-coupled receptors (Hough, 2001; Hattori and Seifert, 2017). Histamine receptors are expressed in both the PNS and CNS (Lindskog, 2017). They differ in their distribution, downstream cellular processes and functions and for that reason, histamine has been shown to have different effects depending on the histamine receptor subtype it is bound to (Ikoma et al., 2006). Histamine receptors belong to the largest membrane proteins family known as G-protein-coupled receptors (GPCRs) family (Panula *et al.*, 2015). GPCRs are characterized by an extracellular N-terminal domain and

intracellular C-terminal domain. The extracellular terminal followed by seven transmembrane α-helical domains connected by 3 intracellular and 3 extracellular loops (Wess, 1997). GPCRs transduce their signals *via* activation and coupling of heterotrimeric G-proteins intracellularly (Panula *et al.*, 2015; O'Mahony, Akdis and Akdis, 2011). These intracellular G-proteins consist of three subunits Gα, Gβ and Gγ subunits, which play a key role in transducing external stimuli into intracellular signaling cascades (O'Mahony, Akdis and Akdis, 2011; Kamato *et al.*, 2015). Until now, there are twenty α subunits, six β subunits and twelve γ subunits identified (Oldham and Hamm, 2008).There are four major subtypes of heterotrimeric G proteins, classified according to Gα subunit; $G_{\alpha i/o}$, $G_{\alpha s}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$ (Sandhu *et al.*, 2019). The $G_{\alpha i/o}$ inhibit the activity of adenlyl cyclase and subseuantily decrease in cAMP formation while the Gαs stimulate the activity of adenlyl cyclase, to result in an increase in cAMP formation. The cAMP consequently activates protein kinase A (PKA) (Sandhu *et al.*, 2019; O'Mahony, Akdis and Akdis, 2011). $G_{\alpha q/11}$-proteins activate PLC, which lead to the hydrolysis of phosphatidylinositol biphosphate (PIP2) in to diacyl glycerol (DAG) and inositol triphosphate (IP3). DAG and IP3 leading to activation of PKC and rise in intracellular Ca^{+2} ions (Marinissen and Gutkind, 2001; O'Mahony, Akdis and Akdis, 2011). $G_{\alpha 12/13}$ proteins can regulate the small G-protein RhoA and Rac small GTPase to control and regulate cell proliferation (Panula *et al.*, 2015; Neves, Ram and Iyengar, 2002).Figure 1.23 summarizes the expression, signaling transduction pathways and biological effect for all four type of HRs.

1.3.2.1 Histamine H_1 receptor (H_1R)

A) Overview

In 1966, H_1R was firstly introduced by Ash and Schild (Ash and Schild, 1966), and was first cloned in 1991 by Yamishita et al., (1991). In 1993 , the gene encoding the human H_1R was cloned by Debacker and co-workers (Debacker *et al.*, 1993). H_1R is expressed predominantly in most brain regions such as neocortex, hippocampus, nucleus accumbens, thalamus, hypothalamus, cerebellum and basal ganglia (Schwartz *et al.*, 1991; Villemagne *et al.*, 1991; Hill, 1990). H_1R apart from the brain and spinal cord is present in smooth muscles in the lung, mucus membrane in the gastrointestinal tract, genitourinary system, cardiovascular system, dorsal root ganglion and primary sensory

neurons (Kashiba and Senba, 2001; Ninkovic and Hunt, 1985; Hill *et al.*, 1997). At a cellular level, H_1R is expressed on various cell types including keratinocytes, mast cells (Greaves and Davies, 1982; Ashida et al., 2001), chondrocytes (Taylor and Woolley, 1987), nerve cells, endothelial cells (Lo and Fan, 1987), neutrophils (Taniguchi, 1991) and dendritic cells (Idzko et al., 2002). H_1R plays important role in eating, drinking energy homeostasis and thermoregulation (Tabarean, 2016). It may also be involved in behaviour, anxiety, learning and memory (Kárpáti *et al.*, 2019; Yanai *et al.*, 1998). Activation of H_1R in airway leads to contraction of smooth muscle cell contraction, increased vascular permeability of endothelial cell and stimulates synthesis of prostacyclin and platelet activating factor (Leurs, Church and Taglialatela, 2002). Clinically, H_1R antagonists are used to treat allergic disorders such as rhinitis, dermatitis, conjunctivitis and can also be used in combination with corticosteroid or anti-leukotrienes for preventing and/or treating allergic asthma (Tatarkiewicz et al., 2019; Bartho and Benko, 2013).

B) Signalling pathways

H_1R is linked to the G-protein-coupled receptors (Hill et al., 1997). It is distinguished by its long third intracellular loop (208 amino acids) and a short (17 amino acids) intracellular C terminal tail (Jongejan *et al.*, 2005; Hill *et al.*, 1997). Stimulation of this receptors causes cascade of downstream mechanisms including stimulation of PLC catalysed hydrolysis of membrane PIP2, producing inositol IP3 and DAG. Subsequently, this leads to activation of PKC at the cell membrane *via* DAG and increased intracellular concentration of Ca^{+2} which forms a complex (Ca^{+2} CaM) of calcium with calmodulin. This complex causes inhibition of K^+ voltage-gated channels type 7 (Kv7 channels) and this results in depolarisation and change in biological action (Brown & Passmore, 2009; Chen et al., 2016). Indeed, stimulation of this receptor has been shown to regulate and control other signaling pathways such as PLA2 and MAP kinase pathway, stimulation of nitric oxide (NO) synthesis (Lipnik-Stangelj, 2006; Bakker *et al.*, 2004; Yang and Hatton, 2002). Finally, activation of the H_1R can stimulate nuclear factor κB (NFκB) by two different ways; first one is mediated by both the Gq/11- and Gβγ-subunits and second one mediated by only Gβγ-subunits *via* constitutively activated of H_1R (Bakker *et al.*, 2001). Activation of nuclear factor κB (NFκB) play an important

role in controlling and regulating the transcription of several genes involved in inflammation and various allergic conditions (Bakker *et al.*, 2001). Figure 1.16, A shows a summary diagram for the most common H_1R signaling pathways.

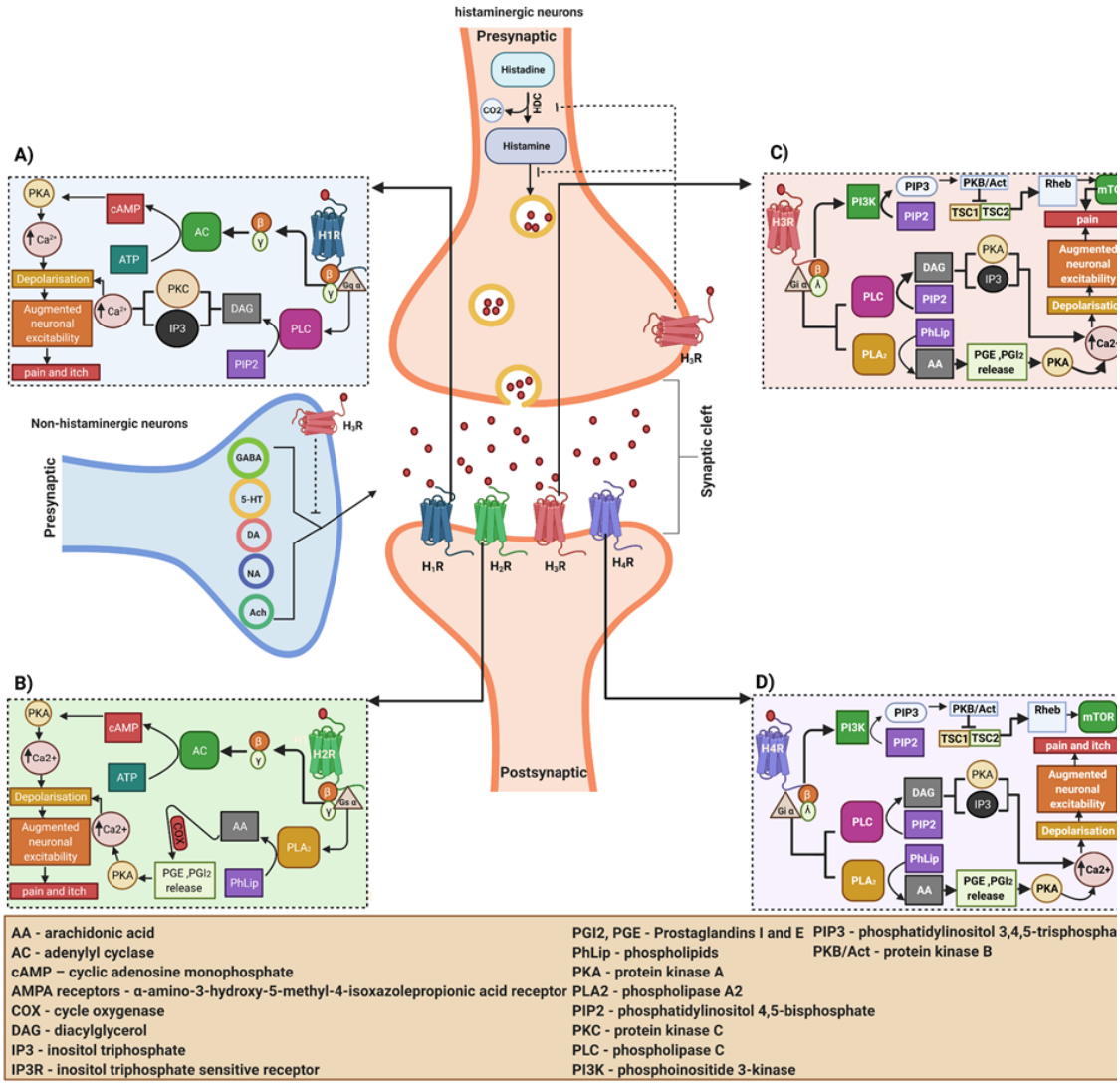


Figure 1.16 A summary diagram for the most common HR signaling pathway. A) H_1R signaling pathway, **B)** H_2R signaling pathway, **C)** H_3R signaling pathway and **D)** H_4R signaling pathway**.** The information in the figure is partially based on Hanuskova and Plevkova, 2013; Branco et al., 2018; Ghamari et al., 2019; Obara et al., 2020.

C) Clinical pharmacology

H_1R is involved in most of the physiological effects caused by an allergic and inflammatory reaction (O'Mahony, Akdis and Akdis, 2011; Togias, 2003). Pre-clinical and clinical trials confirmed that the stimulation of H_1R by using selective agonist cause various effect on both PNS and CNS. In the lung, H_1R causes contraction of the smooth muscles, increase vascular permeability, stimulate synthesis of prostacyclin and platelet

activating and release of nitric oxide (Leurs, Church and Taglialatela, 2002; Carter *et al.*, 1988; O'Mahony, Akdis and Akdis, 2011). Also, H_1R mediates hypersensitivity response of allergic reaction which include sneezing, runny nose, swelling and itching (Togias, 2003). Initially, first-generation H_1R-antihistamines were developed to control allergic condition (anti-allergic drugs)(Church and Church, 2013). First-generation H_1R-antihistamines can cross the blood-brain barrier (BBB) and cause many side effect such as sedation and changes in locomotor activity (Shahid *et al.*, 2009). Additionally, these type of drugs have antimuscarinic and anti-serotonergic effects (Simons, 2004). Later non-sedating H_1R antagonists were developed, they cannot cross the BBB and therefore they produce less side effects comparing to the first-generation (Criado *et al.*, 2010). Several of these second-generation antihistamines have become very successful therapeutics in the treatment of allergic symptoms such as terfenadine, loratadine, desloratadine and certirizine. Figure 1.17 represent the common H_1R ligand used in preclinical and clinical studies.

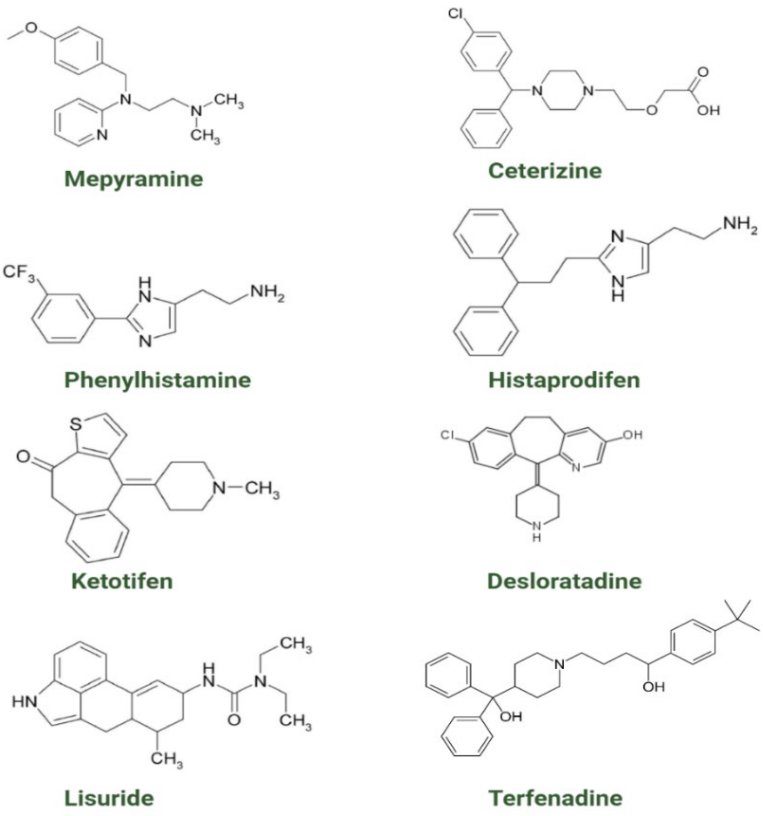


Figure 1.17 Structures of common H_1R ligands used in pre-clinical and clinical studies.

1.3.2.2 Histamine H_2 receptor (H_2R)

A) Overview

Ash and Schild proposed the existence of 2nd type of histamine receptors in 1966 (Ash and Schild, 1966). They found that some antihistamines were not able to block gastric acid secretion mediated by histamine and based on that they proposed presence of another histamine receptor. After six year and exactly in 1972, Black and co-workers discovered the first selective H_2R antagonist (burimamide), which blocked the effect of histamine in smooth muscles of heart and stomach (Black *et al.*, 1972). The distribution of H_2R in mammalian brain is similar to H_1R (Hill et al., 1997). It is widely expressed in the basal ganglia, hippocampus, amygdala, cerebellum, hypothalamus and cerebral cortex (Traiffort *et al.*, 1992; Hill *et al.*, 1997). H_2Rs have also been identified in cardiac tissue, gastrointestinal tract, smooth muscle cells and immune cells (Sander *et al.*, 2006; O'Mahony, Akdis and Akdis, 2011). RT-PCR indicated the expression of H_2R in DRG in mouse (Kajihara *et al.*, 2010) and in the primary afferent neurons (Yue *et al.*, 2014). Stimulation of this type of receptors can promote mucus production from smooth muscles of the airway, increase vascular permeability, and induce stomach acid secretion (Smit *et al.*, 1996; Thangam *et al.*, 2018). In the brain level, the function of H_2R includes modulation of cognitive processes and of sleep-wake cycle. Moreover, H_2R stimulate glucose metabolism and food intake (Schneider et al. 2014). Therapeutically, H_2R antagonists used are to treat gastroesophageal reflux disease and peptic ulcer (Singh, Gohil and Ramírez-García, 2018).

B) Signalling pathways

H_2R is linked to the G-protein-coupled receptors (Hill et al., 1997). Structurally, different than H_1R, it is characterized by short third intracellular loop (30 amino acids) and very long C-terminal tail (70 amino acids). The downstream mechanisms of this type of receptor include activation of Gαs-proteins leading to the stimulation of the adenylyl cyclase and subsequent activation of second messenger cAMP (Panula et al. 2015). Production of cAMP activates protein kinase A (PKA) and subsequent activation of transcription factor CREB, that is responsible for multiple physiological and pathological processes (Del Valle and Gantz, 1997). Also, cAMP-PKA signaling pathway activate Ca^{+2}

channels which cause increase Ca^{+2} influx to the cell (Wellner-Kienitz *et al.*, 2003) and inhibit voltage gated K^+ channel (Kv3.2) leading to reduction of the fast spiking frequency in the neurons (Atzori *et al.*, 2000). Additionally, PKA activates the H^+/K^+-exchanger in the stomach which cause release of the gastric acid secretion(Ahmad *et al.*, 2010). Furthermore, H_2R *via* Gαq/11 proteins can activate PLC-β-IP3/DAG-PKC signalling pathways (Wang *et al.*, 2000). Activation of PKC leads to inhibition of the transient receptor potential cation channels (TRPV) and reduced neuron sensitization (Radresa *et al.*, 2013). Figure (1.16, B) a summary diagram for the most common H_2R signaling pathway.

C) Clinical pharmacology

H_2R is involved in the gastric acid secretion. Pre-clinical and clinical trials established that the activation of H_2R controls and regulates many physiological functions such as contraction of the heart, gastric acid secretion, cell proliferation, differentiation and immune response (Shahid *et al.*, 2010). Several H_2R antagonists, cimetidine being the first, have been used successfully in treatment of gastro-oesophageal reflux disease, gastric and duodenal ulcers (Nash, Lambert and Deakin, 1994; Rackoff *et al.*, 2005). Several of these therapeutic H_2R antagonists such as ranitidine and famotidine have become very popular drugs and generated annual sales of billions (Savarino and Dulbecco, 2004). Indeed, H_2R are also involved in the activation of immune system *via* production of monocytes, dendritic cells, cytokine and chemokine, proliferation of T cell and stimulate synthesis of antibodies (Lichtenstein and Gillespie, 1975; Thangam *et al.*, 2018). Interestingly, using a combination of H_1R and H_2R antagonists were very effective in controlling itch in patient with chronic urticaria (Monroe *et al.*, 1981). Figure 1.18 illustrates the common H_2R ligand used in preclinical and clinical studies.

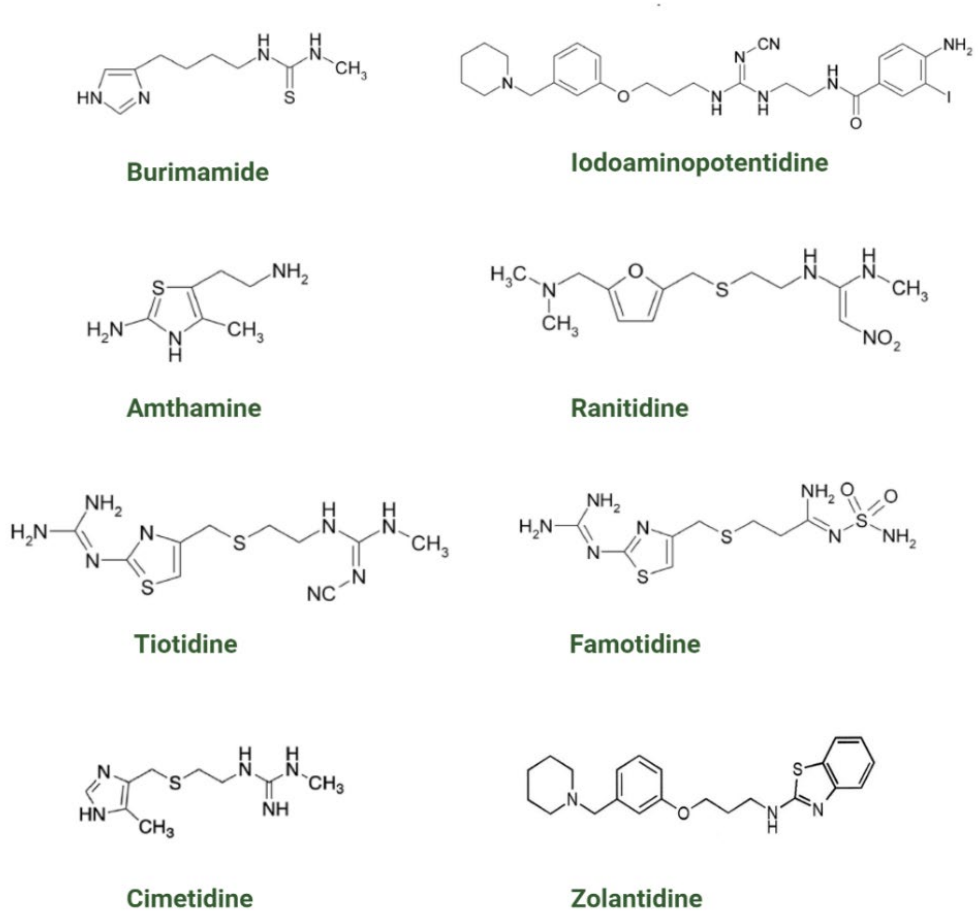


Figure 1.18 Structures of common H_2R ligands used in pre-clinical and clinical studies.

1.3.2.3 Histamine H_3 receptor (H_3R)

A) Overview

In 1983, H_3R was first identified on histaminergic neurones as a novel auto-receptor in the brain (Arrang, Garbarg and Schwartz, 1983b). In 1987, Trzeciakowski found that inhibition of myenteric nerve presynaptically caused contraction of the guinea-pig ileum by a class of receptors that pharmacologically looked like H_3Rs defined previously by Arrang et al. (Schwartz *et al.*, 1990). In 1999, Lovenberg et al., after extensive searches cloned H_3Rs and identified sequences that might be related to GPCRs family in both humans (Lovenberg *et al.*, 1999) rats (Lovenberg *et al.*, 2000). H_3R is expressed almost exclusively in the CNS , however, it can also be detected in other tissues such as cardiac tissue, small intestine, prostate and testis in humans (Lovenberg *et al.*, 1999). In 2007, Cannon et al., also detected H_3R in skin, DRG and spinal cord in rodent by using immunohistochemical localization methods (Cannon *et al.*, 2007). H_3R plays an

important role in various physiological process such as sleep-wake cycle, cognition behavior, locomotor activity, homeostatic of energy levels, metabolic processes and neuro-inflammatory (Dimitriadou *et al.*, 1994; O'Mahony, Akdis and Akdis, 2011). All these outcomes were confirmed using H_3R knockout mice. H_3R knockout mice showed behaviour changes and altered motor activity (Toyota *et al.*, 2002) and, development of different metabolic syndrome such as obesity, hyperphagia, and diabetes mellitus (Tokita, Takahashi and Kotani, 2006; Yoshimoto *et al.*, 2006). In addition, Teuscher et al suggested that H_3R deficiencyin mice may lead to an increase in neuro-inflammatory diseases and can enhance the expression of interferon-γ-inducible protein 10, macrophage inflammatory protein 2 (MIP_2), and chemokine receptor type 3 ($CXCR_3$) (Teuscher *et al.*, 2007). Clinically, numerous H_3R antagonists went through several clinical trials for control of numerous types of CNS disorder such as narcolepsy, Alzheimers's disease and epilepsy. Up to date, only pitolisant (Wakix ®) has been approved by the European Medicines Agency (EMA) for the treatment of narcolepsy (Kollb-Sielecka et al., 2017).

B) Signalling pathways

H_3R is Gαi/o-coupled protein mainly expressed in CNS and act either as auto-receptor, inhibiting the release of histamine or hetero-receptor, inhibiting the release of other neurotransmitter such as such as acetylcholine, noradrenaline, dopamine, or glutamate (Haas et al. 2008). Additionally, there are many publications supporting the expression of this type of receptor in postsynaptic location in different part of brain such as striatum, cerebral cortex, hippocampus, nucleus accumbens, lateral hypothalamus (Pillot *et al.*, 2002; Nieto-Alamilla *et al.*, 2016). Presynaptic H_3R signaling pathways is coupled to Gαi/o proteins (Nieto-Alamilla et al., 2016; Schlicker & Kathmann, 2016). Gαi/o subunit inhibits adenylyl cyclases (AC), which results in reduced intracellular cAMP concentration and subsequent downregulation of PKA (Nieto-Alamilla et al., 2016; Obara et al., 2020). Also, Gαi/o subunit inhibits the activity of the Na^+/H^+ exchanger (NHX) in sympathetic nerve terminals, which leads to increase intracellular Na^+ and then reverse the activity of Na^+/Cl^-dependent neurotransmitter transporters and finally this pathway may leads to inhibition of the release of neurotransmitters such as noradrenaline, dopamine, GABA, glutamate,and serotonin (Silver et al., 2001; Nieto-

Alamilla et al., 2016). Moreover, activation of H_3R and by $G_{\beta\gamma}$ complexes cause decrease the depolarization-induced Ca^{+2} entry *via* inhibition of voltage-gated Ca^{+2} channels and this mechanism is linked to the inhibitory effect of H_3Rs on neurotransmitter release (Zamponi and Currie, 2013; Nieto-Alamilla *et al.*, 2016). Additionally, it was reported that in a subpopulation of striatal neurons with subsequent activation of the IP3-pathway, activation of H_3R stimulates PLC and increased concentration of Ca^{2+} intracellularly (Rivera-Ramírez et al., 2016). The PLC/IP3/ Ca^{2+} pathway is commonly mediated by the Gα subunits, but Gβγ complexes also play a role in the activation of PLCβ (Rebecchi and Pentyala, 2000; Obara *et al.*, 2020; Nieto-Alamilla *et al.*, 2016). Interestingly in transfected cells primary culture neurons of the rat's cerebrocortical and striatal neurons, it was reported that activation of H_3R led to stimulate the phosphorylation of PI3K/Akt/GSK-3β/mTOR signalling pathway (Lai *et al.*, 2016; Obara *et al.*, 2020). These effects are transferred *via* Gi β and γ subunits (Lai *et al.*, 2016). Indeed, H3R activation stimulated activation of the MAPK pathway and this effect mediated by Gβγ complexes (Drutel *et al.*, 2001; Flores-Clemente *et al.*, 2013). H_3R is a potential drug target for the treatment of several important neurologic and psychiatric disorders such as attention deficit hyperactivity disorder, Parkinson's disease, schizophrenia, addiction, obesity, epilepsy and sleep disorders (Schwartz, 2011). Figure 1.16, C shows a summary diagram for the most common H_3R signaling pathways.

C) Clinical pharmacology

After nearly three decades of intensive research and studies, numerous of H_3R inverse agonists/antagonists have advanced in clinical development in the last 5 years. Pitolisant (Wakix ®) was approved by the European Medicines Agency (EMA) for the treatment of excessive daytime sleepiness (EDS) or narcolepsy in adults (Kollb-Sielecka et al., 2017). A number of other compounds developed by many other laboratories have been examined in preclinical studies, these include a series of phase I human PET occupancy trials with PF-03654746 for treatment of cognitive disorders (Wager et al., 2011), PF-03654764 for treatment of allergic rhinitis (North et al., 2014), AZD5213 for treatment of Alzheimer's Disease or mild Cognitive impairment (Jucaite *et al.*, 2013; Hannesdottir *et al.*, 2013), ABT-288 for treatment mild-to-moderate Alzheimer's dementia (Haig *et al.*, 2014), MK-6096 for treatment of major depressive disorder and insomnia (Connor *et al.*,

2016; Connor *et al.*, 2017) and GSK239512 for treatment of mild-to-moderate probable Alzheimer's disease, Schizophrenia and multiple sclerosis (A Grove *et al.*, 2014; Jarskog *et al.*, 2015; Schwartzbach *et al.*, 2017). A new ligands PF-0868087 is being intensively tested in preclinical tests for the treatment of allergic rhinitis and it is expected that it will soon undergo clinical trials. In addation, work presented in this thesis focuses on the role of this selective H_3R ligand in controlling neuropathic pain in the animal model. Figure 1.19 represents the common H_3R ligands used in preclinical and clinical studies.

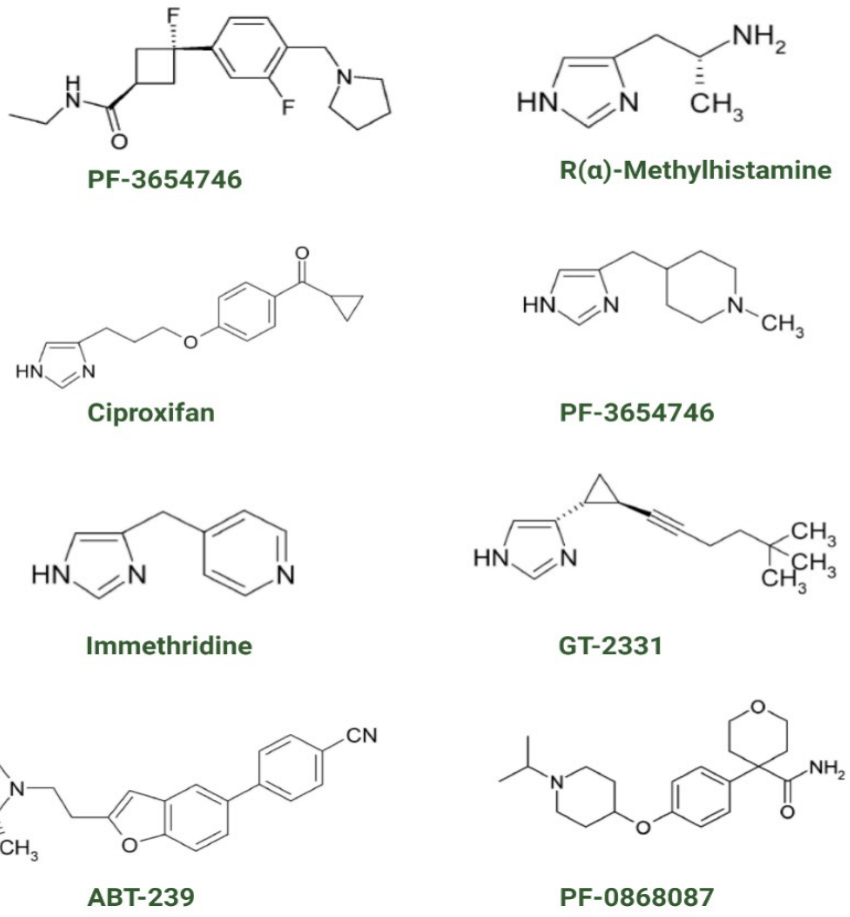


Figure 1.19 S**tructures of common H_3R ligands used in pre-clinical and clinical studies.**

1.3.2.4 Histamine H_4 receptor (H_4R)

A) Overview

H_4R is the most recently recognised histamine receptor. Orphan GPCR played an important role in its discovery at the beginning of the 2000s (Thurmond, 2015; Oda *et al.*, 2000; Liu *et al.*, 2001).The identification of the gene for H_3R provides rich options for biological research for new homology sequences for GPCR. This homology approach

helps to identify receptor showing sequence homology (∼35% identity) to H_3R and with high affinity for histamine (Liu et al., 2001). This new receptor, now known as the histamine H_4R, was described at the same time by other laboratories between 2000 and 2001 (Nakamura *et al.*, 2000; Liu *et al.*, 2001; Oda *et al.*, 2000; Zhu *et al.*, 2001; Nguyen *et al.*, 2001). The homology sequence between humans and various animal species such as mouse, rat, guinea pig, pig, and dog, H_4R is extremely low 65-75% (Liu et al., 2001; Oda et al., 2005; Jiang et al., 2008; Lim et al., 2010). However, the human and monkey H_4R receptors have a higher degree of homology 91 % (Lim et al., 2010). Despite differences between species, the rat, mouse, guinea pig, monkey, and dog H_4R sequences have provided an incredibly valuable tool for comparing the pharmacological characterization of H_4R receptor ligands (Katebe, 2014). H_4R is a chemotactic and mainly expressed on a many types of immune cells such as neutrophils, basophils, eosinophils, T cells, mast cells and dendritic cells of the skin and also on other cells such as the epithelia of the intestinal, spleen lung tissue, synovial tissue, CNS, dorsal root ganglion and primary sensory neurons (Thangam *et al.*, 2018; Thurmond, 2015; Sugata *et al.*, 2007; Rossbach *et al.*, 2011b; Strakhova *et al.*, 2009). H_4R receptor plays a fundamental role in the regulation of the chemo-attraction and recruitment of wide range of immune cells, such as monocyte, eosinophils, mast cells, neutrophils, T cells, and DCs, as well as facilitate cytokine production (Ling *et al.*, 2004; Dunford *et al.*, 2006; Hofstra *et al.*, 2003; O'Reilly *et al.*, 2002). Indeed, H_4R has also been shown to play a role in different autoimmune diseases such as colitis, osteoarthritis, rheumatoid arthritis (Mehta *et al.*, 2020), nociception (Hsieh *et al.*, 2010a; Obara *et al.*, 2020), allergy (Dunford *et al.*, 2006), some types of cancer including colon and breast cancer (Maslinska *et al.*, 2006) and pruritus (Ohsawa and Hirasawa, 2014). Currently, numerous H_4R ligands have been described since the early 2000's. There are few clinical studies on H_4R ligands (Nicoud et al., 2019). At present only H_4R antagonists such as JNJ 38518168 and JNJ 39758979 are currently being evaluated clinically for their potential therapeutic utility in patients with asthma, rheumatoid arthritis, itch and psoriasis (Thangam et al., 2018; Mehta et al., 2020).

B) Signalling pathways

H_4R signaling pathways are coupled to Gαi/o proteins (de Esch *et al.*, 2005; Thangam *et al.*, 2018). Stimulation of this type of receptor causes an inhibition in production of cAMP *via* inhibition of adenylyl cyclase (AC) similar to the H_3R but with different biological effect. Reduction in cAMP leads to a decrease in PKA-mediated downstream signaling (Oda *et al.*, 2000). The reduction in PKA activation leads to phosphorylation of the CREB pathway and increase in mitogen activated protein kinase (MAPK) (Desai and Thurmond, 2011) . In addition, activation of H_4R was found to stimulate Ca^{+2} release from intracellular stores by different pathways (Hofstra *et al.*, 2003; Shahid *et al.*, 2009). For example in mouse mast cells H_4R mediated Ca^{+2} release was found to induce chemotaxis *via* Gαi/o subunits activation (Hofstra *et al.*, 2003) and, in dendritic cells, eosinophils and macrophages cells H_4R mediated Ca^{+2} release was found to induce chemotaxis *via* activation of PLC-IP3- Ca^{+2} pathway started by Gβγ subunits (Ling *et al.*, 2004; Czerner *et al.*, 2014). Figure 1.16, D shows a summary diagram for the most common H_4R signaling pathways.

D) Clinical pharmacology

H4R which was nearly 20 years ago, it has been of great interest to crop researchers and as the subject of extensive research. It is notable that in a short period of time many compounds targeting the H_4R are already entering into the clinical trials for treatment of many diseases. To date many clinical studies have been reported in several disorders such as pruritus, atopic dermatitis, rheumatoid arthritis, asthma, allergic rhinitis and psoriasis (Thurmond et al., 2017a; Thurmond et al., 2017b). H_4R antagonist JNJ 7777120 have been tested in model of asthma , allergic rhinitis and pruritus and it showed anti-inflammatory and anti-pruritic efficacy in preclinical studies (Thurmond et al., 2017a; Thurmond et al., 2017b). Therfore, this compound did not enter into clinical studies due to serious side effects, such as hypoadrenocorticism toxicity as well as short half-life (Thurmond *et al.*, 2014; Thurmond *et al.*, 2017b). Therefore, another analogue of JNJ 7777120 ie., JNJ 39758979, was discovered and it has exhibited anti-pruritic and anti-inflammatory activity with minimal toxicity in animal models (Thurmond *et al.*, 2017b). JNJ 39758979 was introduced into human clinical trails and showed efficacy in

minimising experimental itch in patients suffering from atopic dermatitis (Thurmond et al., 2017b). However, because of the drug-induced agranulocytosis, this compound was withdrawn from the study (Murata et al., 2015) and it was thought that this effect was due to the structure of drug but not due to H_4R antagonism activity (Kollmeier et al., 2014). To overcome the side effect of the drug, another H_4R antagonist 'Toreforant' has been discovered with a different chemical structure (Thurmond et al., 2016). Toreforant has been subjected to clinical trails in patients with asthma, rheumatoid arthritis and psoriasis (Thurmond et al., 2017b). Toreforant was studied in phase 1 human clinical studies to assess safety, pharmacokinetics and pharmacodynamics and according to Thurmond et al, this drug has an excellent oral bioavailability and is safe and well tolerated in phase 1 study in healthy subjects (Thurmond et al., 2017a). There are plenty of H_4R antagonists described in the literature and only a limited number have entered into the stage of clinical trails. However, the clinical data obtained till date indicates that H_4R antagonists could be a valuable source in treating disorders associated with inflammation and allergy such as atopic dermatitis, pruritus, and asthma. Figure 1.20 represents the common H_4R ligands used in preclinical and clinical studies.

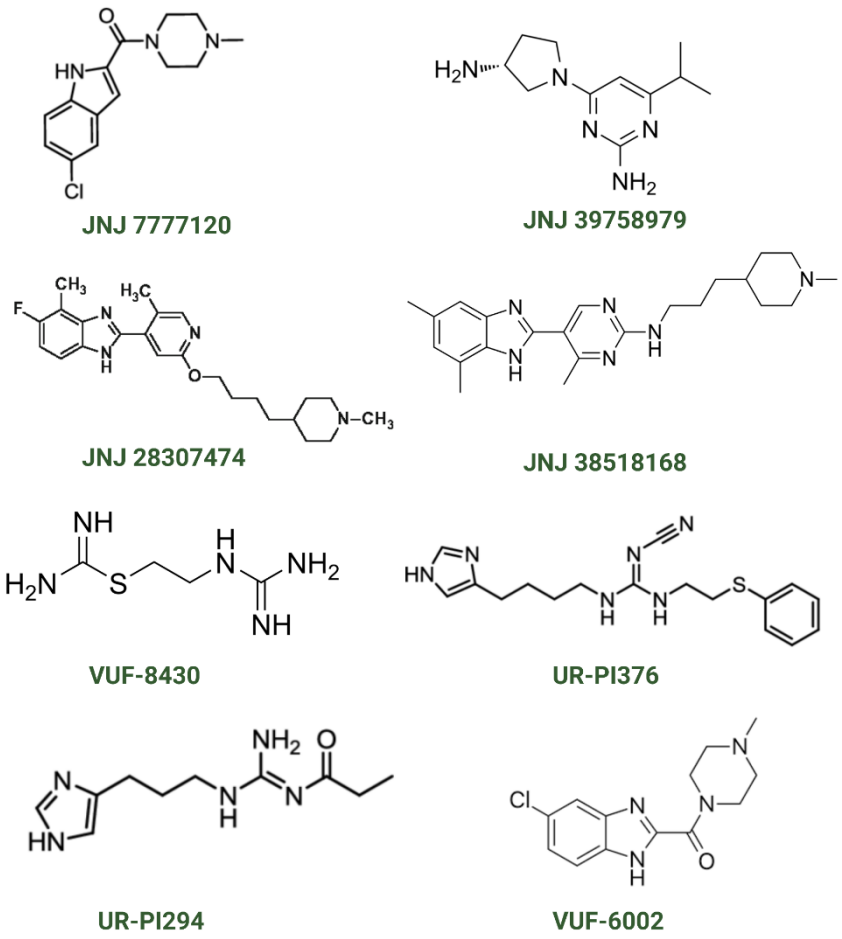


Figure 1.20 Structures of common H_4R ligands used in pre-clinical and clinical studies.

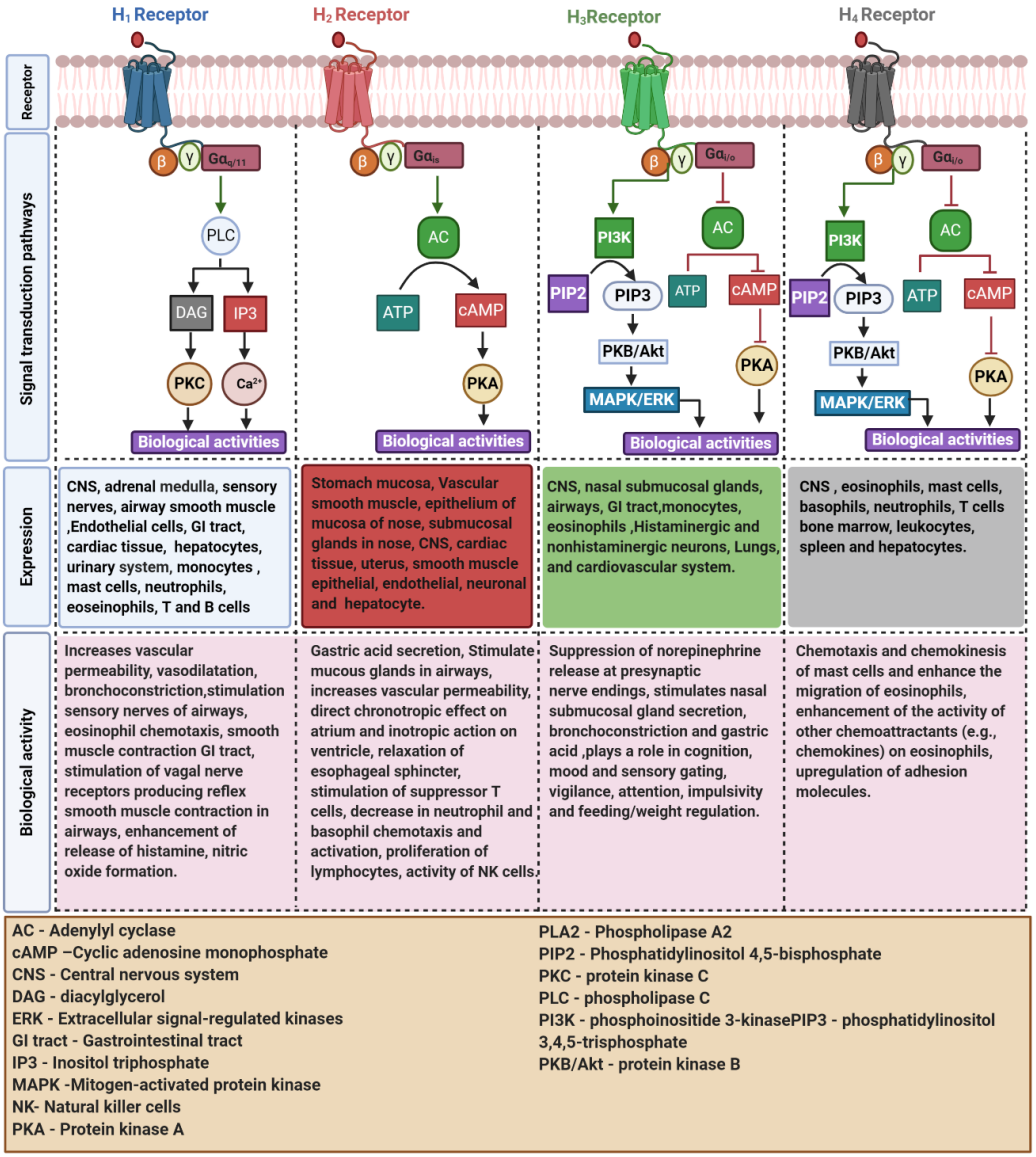


Figure 1.21 Schematic representation of histamine receptors (H_1R–H_4R) in relation to their intracellular signaling, expression and immunological functions. The effects of histamine are mediated by H_1R, H_2R, H_3R, and H_4R which are G protein-coupled histamine receptors. H_1R and H_2R are low-affinity receptors while H_3R and H_4R are high-affinity receptors towards histamine. Signaling through H_1R leads to activation of intracellular *via* Gαq/11, Gαis, and Gαi/o, respectively, while H_4R acts *via* Gαi/o and β-arrestin. Ligands of H_1R–H_4R have different therapeutic applications such as allergic inflammation, gastric acid secretion, neurotransmission, and immunomodulation, respectively. The information in the figure is partially based on Hanuskova and Plevkova, 2013; Branco et al., 2018; Obara et al., 2020.

1.3.3 Role of histamine and histamine receptors in itch and neuropathic pain

As mentioned in the previous sections, itch and pain are two different sensations, even though they share similar neural mechanisms, mediators and receptors (Bíró et al., 2005; Paus et al., 2006). Indeed, and there is complex overlapping between the mechanism of these two conditions (Ständer and Schmelz, 2006b). Several studies were conducted to provide evidence for the involvement of histamine in modulation a broad range of disorders together with itch and pain in both PNS and CNS (Drzezga et al., 2001; Green and Dong, 2016). This modulation start from histaminergic peripheral afferents neurons (C-fibers and Aδ fiber) to first-order neurons of dorsal horn in the spinal cord and second-order neuron which is connecting the dorsal horn up to higher-order cortical and subcortical circuitries (Andrew and Craig, 2001; Chen and Sun, 2020; Khan et al., 2010). Histamine, depending on its concentration, site of action and type of receptors implicated, can both reduce and aggravate sensory perception of itch and pain (Ikoma et al., 2006; Tamaddonfard and Rahimi, 2004). Therefore, histamine and histamine receptors have long been attractive targets for therapeutic interventions in conditions where itch and pain are symptoms requiring therapeutic treatment (O'Donoghue and Tharp, 2005; Obara et al., 2020). Interestingly, it has been shown that histamine can potentially interact with the mechanisms underlying itch and pain, as histamine at low concentrations acts on sensory neurones, produces itching, and at high concentrations can cause pain (Baron et al., 2001; Hough & Rice, 2011; LaMotte et al., 1987; Pini et al., 2016; Simone et al., 1991). In addition, there is literature demonstrating that itch induced by histamine can be converted into the pain such as neuropathic hyperalgesia (Baron et al., 2001). Histamine, in addition to its direct pro-inflammatory effects, also has a variety of other regulatory functions, including regulation of T cells function (Jutel et al., 2001), monocytes (Laszlo et al., 2001), macrophages (Mommert et al., 2021), neutrophils (Hirasawa et al., 2002), and eosinophils (Ling et al., 2004), as well as mast cells (Carlos et al., 2006; Thangam et al., 2018; Branco et al., 2018). It is importanat to note that HRs are expressed not only in a neuronal cell but also in non-neuronal cells such as such as mast cells, T-cell, B-cell, hepatocytes, chondrocytes, monocytes, neutrophils, eosinophils, basophils, dermal dendritic cells, keratinocytes, astrocytes and

blood vessels (Prinz et al., 1999; Borriello et al., 2017; Best et al., 2017; Branco et al., 2018; Thangam et al., 2018). Stimulation of HRs in these non-neuronal cells lead to different pathological condition depending on type and locations of the cells. Interestingly, recent reports also indicate the presence of HRs on mast cells (Branco et al., 2018; Thangam et al., 2018). This non-neuronal localization of HRs supports involvement in the regulation of non-neuronal function related to the modulation of various allergic and inflammatory diseases including itch and pain (Ennis et al., 2013; Sanna et al., 2017; Sanna, Lucarini, et al., 2017). Simons & Akdis, in 2009 found that activation of H_1R on the eosinophil and neutrophil by histamine causes stimulate the release of histamine and other inflammatory increases the expression of adhesion molecules and chemotaxis of those type of cells (Simons & Akdis, 2009). Also, H1R was found to have a crucial role in T cell chemotaxis and increase IFN-γ production (Bryce et al., 2006 ; Ennis et al., 2013). Stimulation of H_2R via histamine inhibits eosinophil, mast cell and neutrophil chemotaxis and enhances production of IL-16 in lymphocytes (Simons & Akdis, 2009; Gangwar et al., 2017). Activation of H_4R induces chemotaxis of mast cells and eosinophil chemotaxis (Hofstra et al., 2003; Akdis and Simons, 2006).Figure 1.22 shows summary of different effects of histamine through its specific receptors on non-neuronal cells. Despite of that effect of the histamine and its receptors on the non-neuronal cells however in this study I will more focus on its role in the primary afferent neurons. Hence, the following section summarizes how the different types of histamine receptor ligands produce their effects on conditions like itch and neuropathic pain.

1.3.3.1 Role of histamine and histamine receptors in itch

For more than a century histamine and its receptors are considered as ‘’gold standard’’ in itch processing in the PNS and CNS (Dong and Dong, 2018). It is now well established from a variety of studies that histamine has been reported to modulate itch through all four subtype receptors.

H_1R has been one of the most extensively studied receptor in the context of itch induced by histamine (Shim and Oh, 2008). Indeed, blockage of H_1R using its specific antagonist (antihistamines) are widely used to control and relieve acute itch symptoms (Lietman *et al.*, 1984; Shim and Oh, 2008).Moreover, local administration of the selective H1R

agonists, evoked scratching behaviour in mouse models of itch (Bell et al., 2004) and, blocking this type of receptor inhibits histamine induce itch (Rossbach et al., 2011). However, the effect of H_1R antagonist is controversial because some studies have argued that the anti-pruritic effect of 1st generation is due to central sedation, rather than antihistamine activity on sensory nerves (Savin et al., 1986; Bell et al., 2004; Imaizumi et al., 2003). Nevertheless, it seems that selective blocking of H_1R, at the minimum, decrease histamine-induced itch, since second generation H_1R antihistamines, which well known as non-sedative are very effective in management of the itch symptoms (Katagiri et al., 2006). Indeed, considerable number of studies has been published supports the involvement of H_1R in controlling acute itch (Fjellner and Hägermark, 1981; Bain, 1951; Hägermark et al., 1979; Sugimoto et al., 1998; Barrett et al., 1985). In 1979, Hagermark et al. reported that mepyramine, H_1R antagonist reduced histamine-induced scratching in humans (Hägermark et al., 1979). In 1985, Barrett et al. showed the same result in rats (Barrett et al., 1985). Also, the same findings have also been reported in mice (Sugimoto et al., 1998). Clinical data confirm that itch caused by local administration of histamine intradermally is mediated *via* the stimulation of the H_1R, since selective antagonists of the H_1R can decrease this response. Pre-treatment with cetirizine, levocetirizine or desloratadine , are highly selective H_1R antagonists before intradermal dose of histamine significantly reduced both score and duration of itch by more than 90% (Denham et al., 2003; Clough, Boutsiouki and Church, 2001; Lahti and Haapaniemi, 1993). Moreover, in 2012 Tanizaki et al found that fexofenadine, which is another highly selective H_1R antagonist, completely suppress histamine-induced itch in human skin (Tanizaki et al., 2012). To further support the role of the H_1R in mediating itch, local administration chlorpheniramine, H_1R antagonist also significantly reduced the scratching induced by local administration of H_1R agonist, 2-methylhistamine (Davies and Greaves, 1980). All these data highlight the major role for the H_1R in mediating histamine-induced itch.

H_2R are noticed in the brain and periphery and the involvement of this receptors in itch is still not completely clear (Buddenkotte and Steinhoff, 2010). As some studies show that H_2R has role (Marks and Greaves, 1977; Kupczyk *et al.*, 2007; Hägermark, Strandberg and Grönneberg, 1979), while others have shown the opposite (Bell,

McQueen and Rees, 2004; Dunford *et al.*, 2007). Indeed, selective H_2R agonist failed to cause scratching in humans or mice and cimetidine, H_2R antagonist failed to inhibit histamine-induced itch in mice (Bell, McQueen and Rees, 2004; Davies and Greaves, 1980; Rossbach et al., 2020). Conversely, targeting H_2R by selective antagonists such as ranitidine or famotidine are used clinically for the control and cure of itchy skin symptoms (Monroe et al., 1981; Watson, Weiss and Harter, 2000). Therefore, most of the current data suggest that H_2R appear to have played a minor role on the itch sensation. More preclinical and clinical studies are needed to confirm and better understand the exact role of peripheral H_2R in itch. Due to many controversial data about the role of H_2R in itch, it is difficult to draw clear-cut conclusions from the all privious studies, For that reason, in the present work we planned to address the role of peripheral H_2R in mediating itch.

H_3R was predominantly expressed in the CNS and was first identified in brain (Arrang, Garbarg and Schwartz, 1983a). There is also evidence for their expression in the PNS and it seems to be an inhibitory receptor comparing to other types of histamine receptors (Cannon *et al.*, 2007; Dong and Dong, 2018). The exact role of H_3R in itch has been debated by researchers. Interestingly, preclinical data suggest that intradermal injection of H_3R antagonists, clobenpropit, evoked scratching behaviors in mice (Hossen *et al.*, 2006; Jeffry, Kim and Chen, 2011). However, this effect of H_3R antagonist could be mediated by H_4R since clobenpropit also has agonistic effect at the H_4R (Oda et al., 2000; Shim and Oh, 2008). It appears the contribution of H_3R to peripherally induced itch transmission is negligeable until now.

H_4R is the most recently discovered subtype, and it is predominantly expressed in immune system cells. At present, there are limited clinical data on the role of the H_4R in mediating histamine-induced itch. However, several animal models showed that the H_4R is implicated in itch responses (Dunford *et al.*, 2007; Thurmond *et al.*, 2010; Thurmond, Gelfand and Dunford, 2008; Shin *et al.*, 2012). In 2007, Dunford et al. found that 4-Methylhistamine, the selective H_4R agonist, has been shown to induce itch in a dose-dependent manner, similar to histamine (Dunford et al., 2007). Interestingly, 4-Methylhistamine- induced pruritus was completely blocked by oral and intraperitoneal dosing of the H_4R antagonist JNJ 7777120 (Dunford et al., 2007; Kamo et al., 2014). A

number of researchers have reported that local administration of H_4R agonists can trigger scratching, which was inhibited by H_4R antagonists (Thurmond et al., 2014; Dunford et al., 2007; Cowart et al., 2008). Moreover, H_4R-deficient mice scratched less in response to histamine and this scratching behaviors was almost completely stopped when H_1R/H_4R antagonists was co-administered (Shim and Oh, 2008; Dunford *et al.*, 2007). All these data indicate the major role for the H_4R in mediating histamine-induced itch.

1.3.3.2 Role of histamine and histamine receptors in neuropathic pain

Histamine has an important role in pain sensation since it is involved in all phases of pain including mechanism transduction, transmission, modulation and perception (Hayashi *et al.*, 2020; Mobarakeh *et al.*, 2000; Obara *et al.*, 2020). A large number of studies have been published exploring the role of histamine in the pain transmission. Interestingly, histamine acts as both a neuromodulator and classical neurotransmitter within the PNS (primary neurons and DRG) as well as in the CNS (spinal cord and brain) (Schwartz et al., 1991; Hough and Rice, 2011b; Mobarakeh et al., 2000; Andrew and Craig, 2001; Obara et al., 2020; Hayashi et al., 2020). Preclinical research demonstrates that histamine has two-way effect according to site and type of the receptors. For example, histamine in CNS work as antinociceptive, while in the PNS it is nociceptive (Yue et al., 2014; Khalilzadeh et al., 2018). Indeed, it was proven that when histamine levels decreased in certain areas of the brain that promote nociceptive behaviour while, oppositely when histamine level increased in brain that promote analgesic effects (Khan et al., 2010; Malmberg-Aiello et al., 1994). Regarding to the role of histamine in controlling acute and chronic pain, multiple behavioural studies have exhibited that the histamine injected directly into various brain areas, such as; somatosensory cortex or hippocampus, resulted in a reduction in pain sensation (Erfanparast et al., 2010; Tamaddonfard & Hamzeh-Gooshchi, 2014). Also, intracerebroventricular (ICV) administrationof histamine blocked mechanical and thermal hypersensitivity, which is connected with neuropathic pain (Sanna et al., 2015; Wei et al., 2016). In addition, several lines of evidence have shown that histamine effects in modulating of pain can vary depending on many factors such as site of action, receptor subtype and concentration of the histamine (Tamaddonfard and Rahimi, 2004, Obara et al., 2020). Indeed, the

antinociceptive effects of different antihistamines are well recognized in different animal models of pain (Raffa, 2001; Farzin et al., 2002; Popiolek-Barczyk et al., 2018). At that time, H_1R ,H_3R and H_4R are believed to be involved in nociceptive transmission (Hough and Rice, 2011; Popiolek-Barczyk et al., 2018), while the involvement of H_2R in the pain sensation, particularly, neuropathic pain are somewhat contradictory (Farzin et al., 2002; Khalilzadeh et al., 2018). Based on this fact several antihistamine agents which are targeting a single subclass of HR have been assessed for control of peripheral neuropathic pain in preclinical and clinical studies (Raffa, 2001).

The antinociceptive effects of H_1R antagonists has been recognized in both preclinical (Malmberg-Aiello et al., 1998; Parada et al., 2001) and clinical studies (Santiago-Palma et al., 2001). In 2001 Parada et al., 2001 found that local administration of H_1R antagonists meclizine or pyrilamine produced an antinociceptive effect in a dose-dependent manner (Parada et al., 2001). Moreover, studies using knockout mice lacking the H_1R showed that these mice demonstrated significantly less responses to nociceptive stimuli when compared to wild-type mice (Mobarakeh et al., 2002). Clinically, majority of trials examined diphenhydramine, 1st generation H_1R antagonist, diphenhydramine for its analgesic potential in the treatment of different type of pain such as dysmenorrhea, head and face pain, trigeminal neuralgia, and thalamic syndrome (Rumore & Schlichting, 1986; Austin and Moalem-Taylor, 2010; Obara et al., 2020). Additionally, diphenhydramine demonstrated that when combined with opioids it acts as a potential analgesic in refractory cancer pain (Fischberg et al., 2001; Obara et al., 2020).

Like H_1R, the involvement of the H_2R in pain perception has been documented previously. Several animal studies have shown that H_2R plays a role in thermal and/or mechanical pain transmission (Mobarakeh *et al.*, 2006; Yanai *et al.*, 2003; Hasanein, 2011). Also, Bluhm et al. reported that several H_2R antagonists potentiated analgesic effect of opioids, while seven year later Gogas et al., proved that H_2R antagonists produced inhibition of antinociceptive effect of morphine in a dose-dependent manner (Gogas *et al.*, 1989; Bluhm, Zsigmond and Winnie, 1982). By contrast, other studies verify that H_2R antagonists caused hypernociception effects (Lamberti *et al.*, 1996). In addition, H_2R antagonists seem to have dual effects on modulate pain sensation across

interaction with opioid system (Gogas et al., 1989; Mobarakeh et al., 2006; Oluyoumi and Hart, 1991). In fact, H_2R antagonists have been reported to potentiate (Bluhm et al., 1982; Gogas et al., 1989) or to inhibit opioid antinociception (Hanig et al., 1981; Hui et al., 1985). It seems that there is conflicting data on the effects of histamine H_2R antagonists on opioid antinociception.

There is some literature availble stating the potential role of H_3R in pain, showing that the pharmacological understanding of these agents in antinociception is not fully understood. As mentioned previously in section 1.3.2.3 H_3R receptors are predominantly expressed within CNS (Lovenberg *et al.*, 1999). However, lately reported data showing the presence of H_3R on neurons, emphasize its contribution in several neuronal functions such as nociceptive transmission (Cannon *et al.*, 2007). In fact, the potential contribution of H_3Rs in pain processing has been proposed. Several studies investigated that both H_3R agonists and antagonists in various animal models of pain. But the data about the effects of H_3R receptor ligands on nociception are somewhat contradictory and the findings on whether pronociceptive or antinociceptive effects occur differ based on type of animal models, the compounds, and site of administration of the tested drugs (Hough and Rice, 2011a; Medhurst *et al.*, 2007; Zhang *et al.*, 2012). Many studies have found that using an H_3R agonist reduces nociceptive response (Hough and Rice, 2011; Rouleau et al., 1997). Conversely, other studies have demonstrated the ability of H_3R antagonists to reduce sensitivity in multiple models of neuropathic pain (Medhurst et al., 2008; McGaraughty et al., 2012). Activation of H_3R by using selective agonists such as imetit and immepip have been shown to cause allodynia (Farzin, Asghari and Nowrouzi, 2002). However, H_3R agonist was able to reduce nociceptive responses in the formalin-induced hyperalgesia (Cannon and Hough, 2005; Chaumette et al., 2018). Also, immepip significantly elevated tail-pinch threshold, this effect was prevented by administration of H_3R antagonist thioperamide (Cannon et al., 2007; Chaumette et al., 2018), Furthermore, previous evidence has shown that various H_3R antagonists reduced mechanical allodynia in animal models of neuropathic pain and pain induced by complete Freund's adjuvant and capsaicin (Medhurst *et al.*, 2007; Hsieh *et al.*, 2010b; Medhurst *et al.*, 2008). This suggests that H_3R antagonists may be more beneficial than a H_3R agonist, although more research is certainly needed to clarify the involvement of

peripheral, spinal and brain H_3R in various pain states.

Regarding H_4R, the antinociceptive effects of the H_4R antagonist JNJ-7777120 have been proposed (Hsieh et al., 2010a). As described previously, H_4R is expressed in many different types of cell in the immune system such as T lymphocytes, mast cells, leukocytes and dendritic cells. Interestingly, the lately reported expression of H_4R on human and rodent CNS emphasizes its involvement in neuronal functions including pain transmission (Sanna *et al.*, 2015). New direct evidence found that targeting H_4R leads to reduces pain perception as it is involved in the early phase of acute inflammation induced by carrageenan in the rat (Connelly *et al.*, 2009) and in persistent inflammatory pain. Therefore, targeting of H4R by selective antagonist JNJ-7777120 showed strong antinociceptive activity in several preclinical model of pain such as complete Freund's adjuvant-induced pain, monoiodoacetate model of osteoarthritis, spinal nerve ligation and sciatic nerve constriction injury models of chronic neuropathic pain, as well as in a skin-incision model of acute postoperative pain (Hsieh et al., 2010a; Tiligada et al., 2011).

I co-authored a review article dedicated to the role of histamine and histamine receptors in neurophathic pain that is included in appendix 1.

All together the above evidence strongly supports the rational to study the role of histaminergic system in itch and chronic pain. Table 1.8 and 1,9 represents a summary of the effects produced by histamine receptor ligands in different animal models of itch and neuropathic pain. A greater understanding of the role of histaminergic system in both conditions will be a valuable tool in developing the new-generation therapy for reducing and control itch and chronic neuropathic pain in human

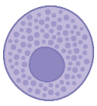
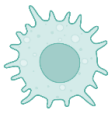
	H_1R	H_2R	H_3R	H_4R
 **Mast Cell**	- Increase chemotaxis. -Increases the Th1 type of cellular immune response and IFN-γ production. -Activation of mast cell. -increase IP3/[Ca2+]. - Stimulates the further release of histamine and other immune mediators	-Inhibits eosinophil,mast cell and neutrophil chemotaxis.	The expression of H_3R appears to be low in peripheral tissues while it is high in neuronal mast cells and eosinophils, and therefore H_3R is not considered an important target in allergy and inflammation	-Increased calcium influx, degranulation, cytokine release. -Increase leukotriene and LTB4 production. -regulate the migration of mast cells
 Macrophages	- Increase chemotaxis. -Regulation of CCL18 production.	-Inhibit chemotaxis. phagocytosis, superoxide anion production. -Increase production of TNFα and IL-12 by macrophages.	Unknown.	-Induced macrophage chemotaxis . -induce phagocytosis.
 Eosinophils	-Induces chemotaxis -increases calcium flux	-Inhibit chemotaxis. -Increase IL-4 and IL-13 production	-Induces chemotaxis (controversial)	-Induces chemotaxis -regulate the migration of eosinophils. - Influences eosinophil cell shape. -Upregulates the expression of adhesion molecules (CD11b and CD54)
 Dendritic Cell	-Promotes Th2 polarisation.	-Promotes Th2 polarisation. -IL-10 secretion. - Inhibit IFNγ and TNFα secretion .	-Induces chemotaxis (controversial)	-Modulates the migration and activation.

Figure 1.22 A summary of different effects of histamine through its specific receptors on non-neuronal cells (Akdis and Simons, 2006; Ennis et al., 2013; Cowden et al., 2010; Gangwar et al., 2017)

Table 1.8. A Summary of some recent studies about the effects of histamine receptors ligands in animal models of itch.

Condition	Histamine Receptor	Drug	Mechanism	Key findings	Reference
Itch	**H_1R**	Fexofenadine	Antagonists	Inhibited scratching behaviour in HR-1 hairless mouse model of AD	(Akamatsu et al., 2006) (Tanizaki et al., 2012).
		Chlorpheniramine	Antagonists	Induced scratching behavior in mice	(Davies and Greaves 1980) (Davies et al., 1979)
		cetirizine	Antagonists	Completely reduce histamine-induced itch in humans	(Levander, St and Hägermark, 1991) (Lahti and Haapaniemi, 1993)
		Olopatadine	Antagonists	Significantly suppressed histamine-induced wheal, flare, and itch, starting 30 minutes after oral administration	(Hashimoto *et al.*, 2010)
	H_3R	Thioperamide AQ0145	Antagonists	Induced scratching behavior in mice	(Sugimoto *et al.*, 2004)
		Iodophenpropit and Clobenpropit	Antagonists	Significant increases in scratching behaviour in both mast cell-deficient and wild-type mice	(Hossen *et al.*, 2003) (Jeffry, Kim and Chen, 2011)
		R-α-methylhistamine	Agonist	Significantly inhibited thioperamide-induced scratching behaviour in ICR mice	(Sugimoto *et al.*, 2004)
		Pitolisant	Antagonists	Increases in scratching behaviour in mice	(Thurmond *et al.*, 2010)
		immethridine	Agonist	Inhibited Pitolisant -induced scratching behaviour in mice	(Thurmond *et al.*, 2010)
	H_4R	JNJ 7777120	Antagonists	Reduced histamine-induced scratching Reduced hapten-induced scratching	(Dunford *et al.*, 2007) (Rossbach *et al.*, 2011a)
		4-Methylhistamine	Agonist	Increases in scratching behaviour in mice	(Dunford *et al.*, 2007)
		VUF 8430	Agonist	Increases in scratching behaviour in mice	(Yang *et al.*, 2016)
		ZPL-3893787	Antagonists	Reducing histamine-mediated itch in healthy human subjects	(Kollmeier *et al.*, 2014)

Table 1.9. A Summary of some recent studies about the effects of histamine receptors ligands in animal models of neuropathic pain.

Condition	Histamine Receptor	Drug	Mechanism	Key findings	Reference
Neuropathic Pain	**H_1R**	Fexofenadine	antagonists	Significantly attenuated the mechanical allodynia	(Khalilzadeh *et al.*, 2018)
		Chlorpheniramine	antagonists	Significantly attenuated cold allodynia and decreased mechanical allodynia.	(Khalilzadeh *et al.*, 2018)
	H_2R	Fexofenadine	antagonists	Significantly attenuated the mechanical allodynia	(Khalilzadeh *et al.*, 2018)
		Ranitidine	antagonists	Attenuate mechanical and cold allodynia	(Khalilzadeh *et al.*, 2018)
		GSK207040	antagonists	Blocked the secondary mechanical allodynia in the capsaicin-induced model of pain	(Medhurst *et al.*, 2008)
	H_3R	GSK189254 GSK334429	antagonists	GSK189254 and GSK334429 significantly reversed CCI-induced allodynia and GSK189254 also reversed CCI-induced mechanical hyperalgesia	(Medhurst *et al.*, 2008) (McGaraughty *et al.*, 2012)
		A-96065	antagonists	A-960656 produced a significant reversal of nerve injury-induced tactile hypersensitivity in the rat SNL model of neuropathic pain	(Cowart *et al.*, 2012)
		GSK207040 GSK334429	antagonists	GSK207040 and GSK334429 significantly reversed capsaicin-induced reductions in paw withdrawal threshold	(Medhurst *et al.*, 2007)
		E-162	antagonists	E-162 attenuated nociceptive responses and profound morphine analgesia in males male with CCI.	(Popiolek-Barczyk *et al.*, 2018)
		S38093	antagonists	S38093 exhibited a significant antihyperalgesic effect in the Streptozocin-induced diabetic (STZ) neuropathy model and, in the chronic constriction injury (CCI) model	(Chaumette *et al.*, 2018)
	H_4R	JNJ 7777120	antagonists	Significant anti-nociception in animal model of neuropathic pain.	(Hsieh *et al.*, 2010a)
		VUF 8430	agonist	VUF 8430 showed significantly reduced mechanical hyperalgesia following peripheral nerve injury.	(Smith *et al.*, 2007b)
		TR-7	antagonists	TR-7 attenuated nociceptive responses and profound morphine analgesia in male with CCI.	(Popiolek-Barczyk *et al.*, 2018)

1.4 The mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) has been discovered to be a novel target for the effective control of chronic pain and itch (Obara et al., 2013; Um et al., 2019 ; Kim et al., 2020). Previous research has shown that mTOR inhibition at the level of the peripheral nervous system can decrease chronic pain and histamine-induced itch (Obara et al., 2013 ; Obara and Hunt, 2014). A subset of fibres that respond specifically to cold and itch have been found to contain activated mTOR (Obara et al., 2013). As a strategy for effective pain control in Chapter 7 of this thesis I will focused on the link between mTOR and H_3R pathways in chronic neuropathic pain. Therfore, here I will discuss briefly what is known about mTOR, components, functions, upstream and downstream signalling of mTOR receptors and its role in both acute itch and chronic pain in the following section.

1.4.1 Background

The mammalian (or mechanistic) target of rapamycin (mTOR) is a 289-kDa serine/threonine protein that belongs to member of the phosphoinositide 3- kinase-related kinase (PI3K) family (Brown *et al.*, 1994; Sabatini *et al.*, 1994). It was initially discovered in yeast in the early 1990 (Laplante and Sabatini, 2012). This protein is expressed in all eukaryotic cell as well as neural cells and responsible for the wide range of physiological and pathological processes (Swiech *et al.*, 2008; Laplante and Sabatini, 2012). Figure 1.23 represent the key cellular processes regulated by mTOR protein complexes (mTORC1 and mTORC2). This protein is the target of a lipophilic macrolide compound named rapamycin or sirolimus, which is produced by specific type of bacteria called *Streptomyces hygroscopicus* This type of bacteria is isolated for the first time in the 1970s in a soil sample come from Easter Island in the Pacific Ocean (Sehgal, 2003; Ryskalin *et al.*, 2017). Rapamycin (sirolimus) is a macrolide broadly used as an immunosuppressant with antitumor activity (Martel et al., 1977). It also has anti-proliferative and anti-angiogenic activity (Rao, Buckner and Sarkaria, 2004). Above all, rapamycin, as an autophagy stimulator, can modulate cellular autophagy, thereby modifying the balance of proliferation and survival of the cell.

mTOR plays a vital role in a wide range of body processes and biological functions, such as cell growth, metabolism, proliferation and synaptic plasticity (Caron et al., 2010; Laplante and Sabatini, 2012). This protein plays a central role in neural development and differentiation and therefore plays a pivotal role in the regulation of learning processes and memory improvement (Garza-Lombo and Gonsebatt, 2016). On the other hand, overexpression of mTOR pathway over time has been linked to several pathological conditions. An upregulation of mTOR is known to cause cellular stress and damage accumulation, which leads to a decrease in cell function and promotes the development of a number of pathological conditions such as obesity, diabetes, cancer, aging and neurodegeneration diseases (Um et al, 2004; Hara et al., 2006; Guertin et al., 2009; Zoncu, et al., 2011; Laplante and Sabatini 2012; Saxton and Sabatini, 2017).

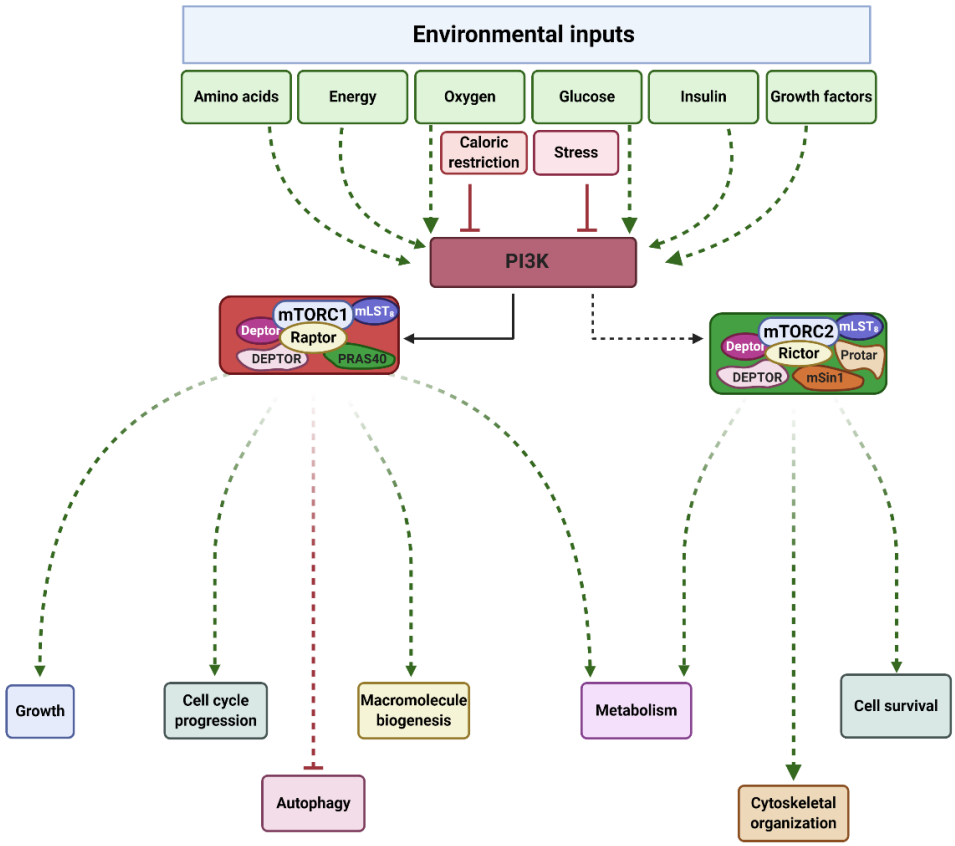
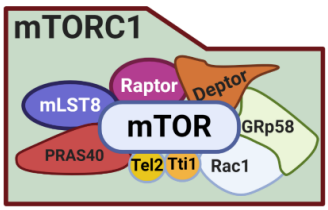
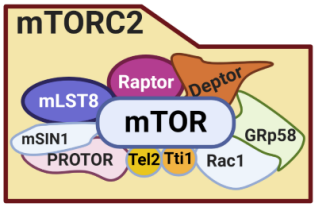


Figure 1.23 The two distinct mTOR protein complexes (mTORC1 and mTORC2) and the regulation of key cellular processes. mTORC1 promotes cell growth, cell cycle progression and proliferation in response to different signals such as growth factors, oxygen, energy levels and nutrients. While mTORC2 responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton. (adapted from Laplante and Sabatini, 2012; Li, Kim and Blenis, 2014; Xu et al., 2020)

1.4.2 Components and functions of mTORC1 and mTORC2 proteins

TOR interacts with other proteins to form two distinct multi-component complexes: the mammalian target of rapamycin complex 1 (mTORC1), which has about eight known proteins, and the mammalian target of rapamycin complex 2 (mTORC2), which has nine known proteins. Figure 1.24 illustrates components of both mTOR complexes; mTORC1 and mTORC2, as well as the functions of each proteins. These two complexes have different upstream regulators and downstream functions (Laplante and Sabatini, 2012). Both mTORC1 and mTORC2 are consist of 6 similar proteins which are mammalian target of rapamycin protein (mTOR), mammalian Lethal with Sec13 protein 8 (mLST8), also identified as GβL (Gβ-like protein) (Kim *et al.*, 2003), DEP (dishevelled, egl-10, pleckstrin) domain-containing mTOR interacting protein (DEPTOR)(Peterson *et al.*, 2009), telomere maintenance 2 (Tel2), Tel2 interacting protein 1 (Tti1) (Kaizuka *et al.*, 2010), 58 KDa glucose-regulated protei (GRp58) also known as ERp57 (Khanal and Nemere, 2007) and Rac1 [Ras (rat sarcoma)-related C3 botulinum toxin substrate 1] (Saci, Cantley and Carpenter. 2011). The specific proteins components of mTORC1 are regulatory-associated protein of mTOR (RAPTOR) (Hara *et al.*, 2002; Yonezawa *et al.*, 2004; Kim *et al.*, 2002) and PRAS40 (Prorich Akt substrate of 40 kDa) (Wiza, Nascimento and Ouwens, 2012; Wang *et al.*, 2007; Thedieck *et al.*, 2007). On the other hand, the specific components of mTORC2 are rapamycin-insensitive companion of TOR (RICTOR) (Sarbassov *et al.*, 2004; Hresko and Mueckler, 2005), mammalian stress activated protein kinase interacting protein 1 (mSIN1)(Frias et al., 2006; Lu et al., 2011; Yao et al., 2017) and protein observed with RICTOR (PROTOR) (Pearce et al., 2007; Pearce et al., 2011; Holmes et al., 2012).





Component	Function
Raptor	An essential component of mTORC1,scaffolding protein important for mTORC1 assembly, stability, substrate specificity and regulation. Also, it is responsible for the recruitment of mTOR downstream targets, such as S6Ks and 4EBPs (Kim et al., 2002).
Deptor	Neatively regulates the activity of mTOR (Sancak Y et al. 2008).
GRp58	Regulates protein-protein interactions via a redox mechanism based on the activity of its two thioredoxin-like domains (Khanal RC et al. 2007)
PRAS40	Neatively regulates the activity of mTOR (Sancak Y et al. 2008)
mLST8	Is essential for the integrity and activation of mTORC (Guertin, D.et al, 2006)
Tel2 and Tit1	Important for mTOR stability and assembly of the mTOR complex and maintain their activities. (Kaizuka T. et al 2010)
Rac1	Positive regulator of mTOR. (Saci A et al. 2011)

Component	Function
Raptor	An essential component of mTORC1,scaffolding protein important for mTORC1 assembly, stability, substrate specificity and regulation. Also, it is responsible for the recruitment of mTOR downstream targets, such as S6Ks and 4EBPs (Kim et al., 2002).
Deptor	Neatively regulates the activity of mTOR (Sancak Y et al. 2008).
GRp58	Regulates protein-protein interactions via a redox mechanism based on the activity of its two thioredoxin-like domains (Khanal RC et al. 2007)
PROTOR	Dispensable for the assembly of other subunits into mTORC2, although it is required for the activation of specific substrates such as SGK1 (Pearce et al. 2007)
Tel2 and Tti1	Important for mTOR stability and assembly of the mTOR complex and maintain their activities. (Kaizuka T. et al 2010)
Rac1	Positive regulator of mTOR. (Saci A et al. 2011)
mLST8	Is essential for the integrity and activation of mTORC (Guertin, D.et al, 2006)
mSIN1	Essential component required for complex formation and kinase activity (Yang Q et al, 2006)

Deptor: DEP domain-containing mTOR-interacting protein
GRp58: 58 KDa glucose-regulated protein
mLST8: Mammalian lethal with sec-13 protein 8
mSIN1: Mammalian stress-activated protein kinase-interaction protein 1
mTOR: Mammalian Target of Rapamycin
mTORC1: Mammalian Target of Rapamycin Complex 1
mTORC2: Mammalian Target of Rapamycin Complex 2
PROTOR: protein observed with rictor
Rac1: Ras (rat sarcoma)-related C3 botulinum toxin substrate 1
Raptor: regulatory-associated protein of mTOR.
Tel2 and Tti1: Tel 2 interacting protein 1

Figure 1.24 Components and functions of mTORC1 and mTORC2 proteins. mTORC1 consists of mTOR, Raptor, PRAS40, mLST8 and Deptor. mLST8 binds to the mTOR kinase domain in both complexes, and it is crucial for their assembly. Deptor acts as an inhibitor of both complexes. Other protein partners differ between the two complexes. mTORC2 contains Rictor, mSIN1, and Protor1. (adapted from Kosach et al., 2015; Takei and Nawa, 2014).

1.4.3 Upstream and downstream signalling of mTOR

mTORC1 and mTORC2 have have different upstream regulators and downstream functions (Laplante and Sabatini, 2012). For example, under the normal activation of mTORC1 pathways by growth factors, exercises, energy status, oxygen and amino acids, these promote cell growth and proliferation *via* the activation of protein and lipid synthesis, which negatively regulate autophagy, whereas mTORC2 controls cell survival and the cytoskeleton system (Laplante and Sabatini, 2012; Karar and Maity, 2011).

As summarised in Figure 1.25, growth factors activate RTKs and the downstream signalling cascades PI3K/AKT and Ras/Erk, resulting in the inhibition of the $TSC_{1/2}$

complex, the most essential upstream negative regulator of mTORC1. In the case of low energy level or hypoxia, the TSC complexes is activated, which lead to downregulation of the protein synthesis. Also, mTORC1 directly and indirectly inhibited AMP-activated protein kinase (AMPK) by phosphorylating RAPTOR and TSC2 respectively. mTORC2 is also activated by same growth factor/RTK/PI3K signalling pathway (Yang et al., 2014). This pathway cascade leads to the control of cell survival, metabolism, and cytoskeletal organisation *via* AGC family kinases (Yang et al., 2014). mTOR1 actives the downstream signalling molecules known as ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP) (Yang et al., 2014). mTORC1 downstream effector also inhibits IRS and mTORC2 *via* inhibitory phosphorylation. mTORC1 phosphorylates 4E-BP which leads to its release from eIF4E and therefore this enhanced binding of eIF4E protein, into eIF4F complex to initiate cap-dependent translation. This means activation of the mTORC1 causes inhibition of 4E-BP and activation of S6K (downstream signalling of mTORC1), with both promoting protein and lipid synthesis (Yang et al., 2014). Several lines of evidence suggest S6K and 4E-BP control cell growth and proliferation in different ways S6K controls the size of the cell but not cell-division cycle, whereas 4E-BP controls cell proliferation but has no effect on the cell size (Ohanna et al., 2005; Dowling et al., 2010). Recently, mTORC1 and its downstream pathways are one of the therapeutic targets for enhancing axon regeneration and functional recovery after neural injury (Yang et al., 2014).

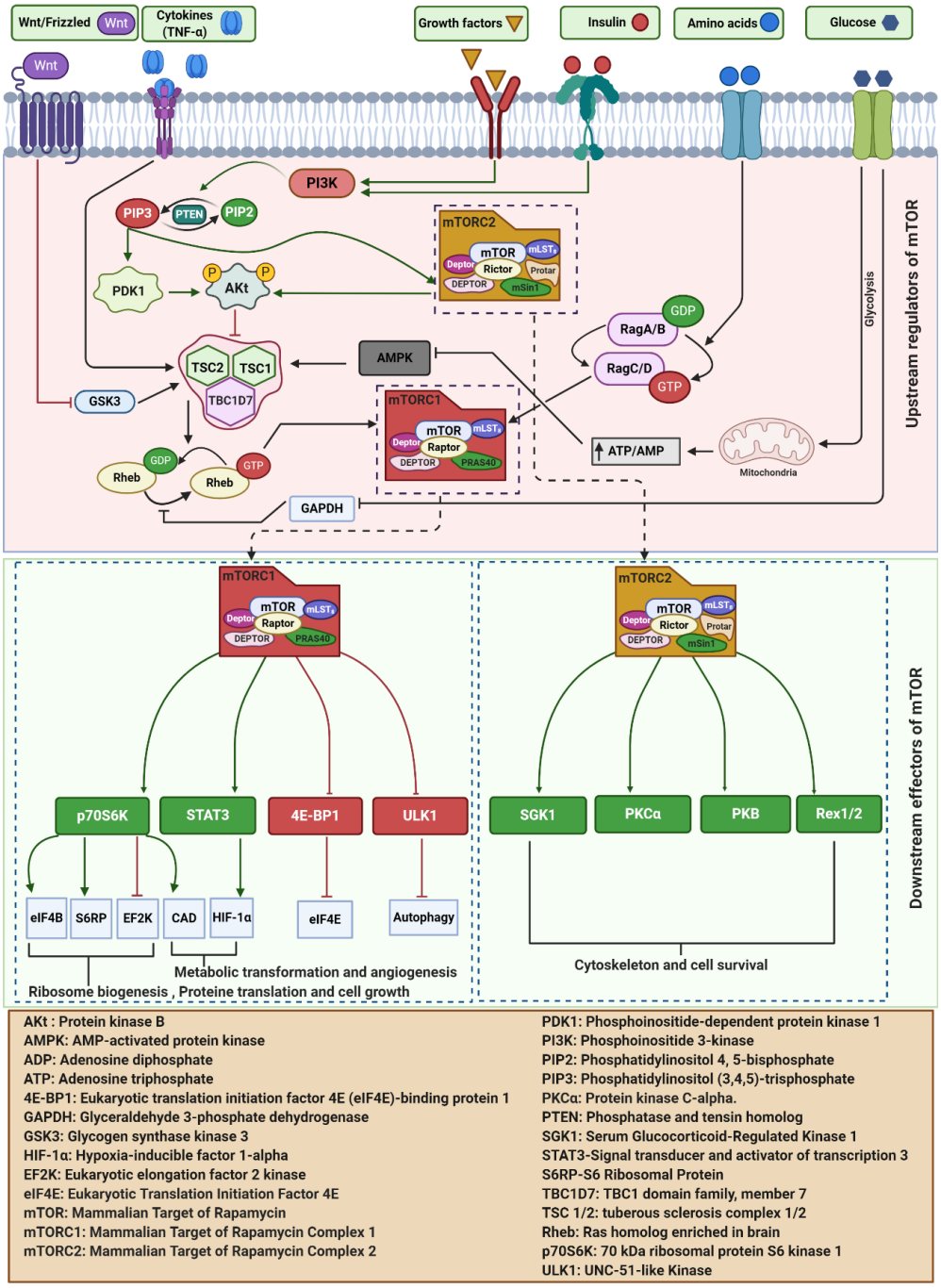


Figure 1.25 Schematic diagram of upstream and downstream of mTORCs. Stimulation of several receptors at the plasma membrane by mitogens, trophic factors and neurotransmitters leads to mTOR activation *via* Ras- and PI3K-dependent pathways. The activity of mTOR can be specifically inhibited by the drug rapamycin. Arrows indicate activation and bars indicate inhibition (adapted from Takei et al., 2004; Swiech et al., 2008).

1.4.4 Role of mTORC1 in pain and itch processing

It is important to note that in relation to pain, several observations support the role of the mTOR, in particular complex1 pathway in chronic inflammatory and neuropathic pain processing (Liang *et al.*, 2013; Obara *et al.*, 2011; Lutz *et al.*, 2015). As explained in section 1.2, that neuropathic pain is the result of lesions or injury in the somatosensory nervous system, which lead to changes in function and structure of the neurons (Shaw, Lanius and van den Doel, 1994). These changes in neural plasticity involving pre and post- translational alteration include changes in the numbers or morphology of neurons, gene expression, functions of receptors, neurotransmission release, sodium channel trafficking (Osborne, Anastakis and Davis, 2018; Theile and Cummins, 2011; Lisi *et al.*, 2015) have been demonstrated to be involve in itch and pain modulation (Sator-Katzenschlager, 2014; Obara et al., 2015; Kwon et al., 2017). This plasticity of neuronal occurred in both structural and functional levels of the neuron in PNS and CNS during neuropathic pain (Luongo *et al.*, 2015; Kwon *et al.*, 2017).

In case of itch, It has been shown that the mTOR pathway is implicated in the pathogenesis of several skin disorders such as melanoma, sclerosis tuberose, psoriasis, acne, wound healing and recently itch (Balato et al., 2014; Obara et al., 2015). It has been indicated that rapamycin, a well-known second-generation immunosuppressive agent, inhibited the activation of mTOR, induced a clinical improvement of skin lesions in treated and reduction in scratching behaviour (Lusková and Dráber, 2004; Cacciapuoti S, 2019). In 2015, Obara et al found that mTORC1 signaling pathway plays an important role in control both histamine-dependent and histamine-independent itch (Obara *et al.*, 2015).

In case of itch, pre-clinical research over the last ten years has shown that mTORC1-mediated ongoing local translation of mRNA maintains the sensitivity of a subset of A-nociceptors thus offering a therapeutic target for the control of some critical components of chronic pain (Geranton et al., 2009; Jimenez-Diaz et al., 2008; Obara et al., 2011). For example, the intrathecal administration of rapamycin mTORC1 inhibitors or systemic delivery of CCI-779, which is a rapamycin derivative, ATP-competitive

mTORC1/2 inhibitors (Torin1) or activators of AMPK (Metformin) blocked the activity of mTORC1 both in sensory axons and in the spinal cord and alleviated hypersensitivity in peripheral neuropathic pain (Norsted et al., 2010; Melemedjian et al., 2011,Obara et al., 2011). Furthermore, it has been confirmed that *in vivo* activation of mTORC1 pathway lead to increase the sensitivity of fast nociceptors conduction and inhibition of this pathway leads to a decrease in the sensitivity of nociceptors (Jimenez-Diaz et al., 2008). In 2013, Luy et al. found that the mTORC1 signalling pathway controls both central and peripheral pain sensation through S6K, by controlling synaptic plasticity in the hippocampus. Indeed, recent studies have indicated the involvement of the insular cortex in mTORC1-mediated analgesia in peripheral neuropathic pain (Kwon et al., 2017).

According to the previous research, it seems that mTOR pathway is involved in the pathogenesis of several dermatological and neural conditions. All the evidence support the idea that mTOR pathway plays an important role in chronic itch and neuropathic pain. Further study will be necessary to evaluate the potential role of mTOR as therapeutic target in order to develop new therapies for both conditions in human.

1.5 Crosstalk between the histaminergic system and mTORC1 pathway

Stimulation of the H_3R leads to inhibition of the production of histamine and its subsequent release from synaptic vesicles of the neurons (Arrang et al., 1983). Furthermore, histamine functions as a heteroreceptor and controls the release of other neurotransmitters, such as; serotonin (Schlicker et al., 1988), norepinephrine (Schlicker et al., 1989), acetylcholine (Clapham and Kilpatrick, 1992) and dopamine (Schlicker et al., 1993).

Interestingly, recent research carried out by Yan and colleagues (2014), found that inhibition of H_3R, caused inhibition of the Akt/GSK-3β pathway, which subsequently inhibits mTORC1 activity (Yan et al., 2014). Therefore, H_3R is potentially involved in the regulation of pain perception through a mechanism directly involving inhibition of the mTORC1 pathway. Figure 1.26 shows schematic diagram of the interactions between H_3R and mTORC1. One of the aime of this thesis is to determine mechanisms that drive the antinociceptive effects of H_3R , with a foucus on mTORC1 signalling pathway.

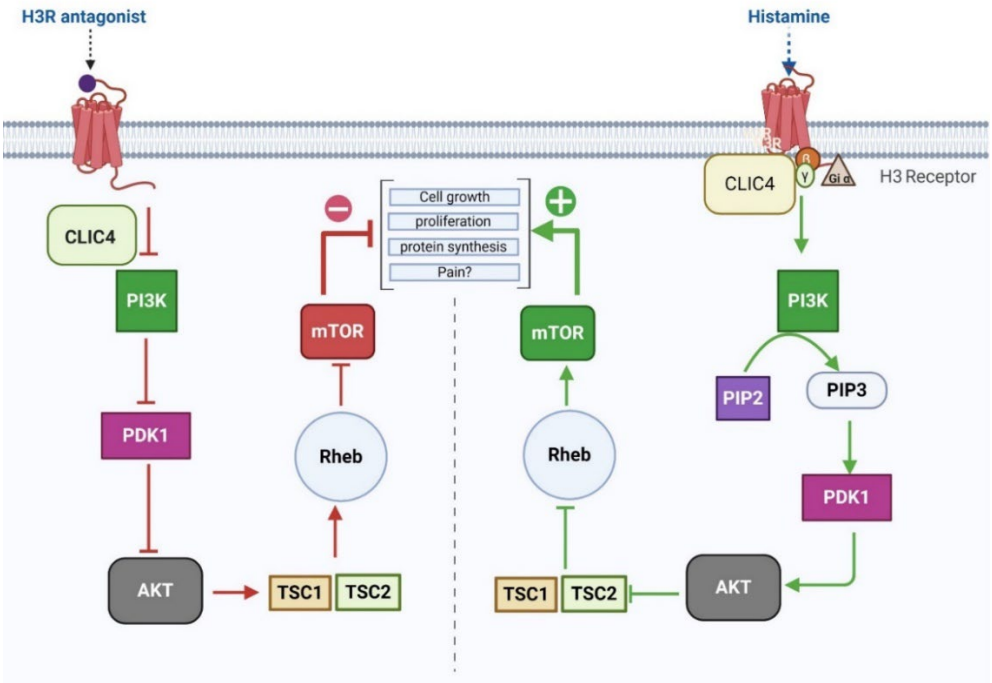


Figure 1.26 Schematic diagram showing the interactions between H_3R and mTORC1. The H_3R antagonist binds with H_3R, causing release of the CLIC4 molecule. This process leads to inhibition of the PI3K/Akt/GSK-3β/mTOR signalling pathway. This has the potential to reduce pain perception (adapted from Yan et al., 2014).

1.6 Aims and hypotheses

Histamine which is present in various cell types of both humans and animals, is arguably one of the most pleiotropic molecules. It is an endogenous ligand for H_1R-H_4R that have been identified in sensory pathways regulating both nociceptive and itch transmission. Neuropathic pain resulting from nerve injury as well as acute itch, where histamine has been an important mediator, have both limited therapeutic strategies due to not completely understood mechanisms underlying these conditions. The recent development of novel ligands targeting the histamine system has provided thought-provoking tools to investigate the role of histamine and its receptors in the regulation of these conditions. Work presented here, further explored novel therapeutic indications based on targeting of the peripheral histamine system using two different mechanisms:

1) direct blocking of H_3Rs using H_3R antagonist PF-0868087; and **2)** scavenging of endogenous histamine by Votucalis (rEV131). Both approaches were investgated using mouse models of acute itch and chronic neuropathic pain. This work also investigated and identified the role of H_1R, H_2R and H_4R in acute itch.

Specific aims and objectives:

1) Further explore the role of histamine and its receptors in the regulation of acute itch and neuropathic pain by using a novel histamine-binding protein Votucalis and scavenging peripherally released histamine.
2) Determined potential for *in vivo* use of novel photoswithable compounds targeting H_4Rs in acute itch.
3) Identify the role of peripheral H_3Rs in the regulation of mechanisms underlying the maintance of neuropathic pain by using a novel CNS-sparing H_3R antagonist PF-0868087.
4) Determine mechanisms that drive the antinociceptive effects of PF-0868087, with a foucus on mTORC1 signalling pathway.

2. GENERAL MATERIALS AND METHODS

This chapter of the thesis describes all the general methods applicable to all *in vivo* and *in vitro* experiments. The *in vivo* behavioral models include induction of scratching behavior by localized injection of pruritic compounds and neuropathic pain model by using the chronic constriction injury (CCI) method. Assessment of scratching behaviors included analysis of videos collected during experiments, while assessment of neuropathic pain was based on using of von Frey and Hargreaves tests.

The *in vitro* biochemical assay analysis includes immunoblotting (Western blot). More specific details including materials, chemical and reagents used in each experiment with the study protocols are described in the methods section of the relevant chapters.

2.1 Ethical consideration

Experimental protocols were approved by the Home Office and were consistent with the guidelines provided by the UK Animals (Scientific Procedures) Act 1986 and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. All experiments were conducted under Project Licence numbers: P8E3496FA and P6694C943 issued to Dr. Ilona Obara and, under Personal Licence I0BDD9511 issued to myself. In addition, the terms and conditions approved by the Comparative Biology Centre (CBC) at Newcastle University were followed.

The use of animals in this thesis was necessary to explore the underlying mechanisms involved in the development of acute itch and neuropathic pain and were important for the development of novel efficacious anti-pruritic and analgesic medications. The animals used for experiments described in this thesis were housed in licensed animal facilities within the Comparative Biology Centre (CBC) at Newcastle University. All efforts were made to minimize animal suffering and to reduce the number of animals used in the study.

2.2 Subjects

Adult male C57BL/6J mice (8 weeks of age; 20–25 g; Charles River Laboratories, Harlow, UK) were allowed to acclimatize to the colony room (Comparative Biology Centre, Newcastle University, UK) for at least 7 days after arrival, and prior to experiments. Four mice were housed in each standard polyethylene cage at a temperature of 21 ± 2°C and humidity of 55% and maintained on a 12-h day/night cycle (lights on at 8:00 A.M.; lights off at 8:00 P.M.). Standard laboratory rodent food and water were available *ad libitum*. For at least 3-4 days before experiments, animals were habituated to testing conditions. The handling and testing of the animals were conducted during the light phase, between 9:00 A.M. and 16:00 P.M. To avoid the confounding effects of the oestrus cycle on itch and pain, and to align the study with other studies conducted by our group, only male animals were used despite evidence showing that sex hormones have an effect on mast cell behaviour, itch and pain sensation (Mogil, 2012; Zierau et al., 2012).

2.3 Drugs used in the experiments

Table 2.1 represents all drugs that were used in different chapters of this thesis. Table 2.2 represents drugs doses, concentration and molecular weight for all drugs that were used in this thesis. In the methods section of relevant chapters, more specific details including preparation, route of administration, materials, chemicals and reagents used in each experiment including the study protocols are described.

Table 2.1 Drugs used in the experiments

Drug	Effects	Supplier	Chapter
Votucalis	Histamine-binding proteins	Akari Therapeutics Plc	**3 and 5**
Mepyramine maleate	H_1R antagonist	Sigma-Aldrich	**3**
Ranitidine hydrochloride	H_2R antagonist	Tocris Bioscience	**3**
JNJ 7777120	H_4R antagonist	Tocris Bioscience	**3 and 4**
Compound 48/80	Mediating histamine-dependent itch	Sigma-Aldrich	**3**
Chloroquine diphosphate	Mediating histamine-independent itch	Sigma-Aldrich	**3**
Histamine	HR agonist	Sigma-Aldrich	**4**
***trans*-VUF16129**	H_4R agonist	Professor Rob Leurs*	**4**
***cis*- VUF16129**	Weak H_4R agonist	Professor Rob Leurs*	**4**
Bavisant	H_3R agonist	Professor Rob Leurs *	**4**
PF-0868087	H_3R antagonist	Ziarco Pharma	**6 and 7**
Immepip dihydrobromide	H_3R agonist	Tocris Bioscience	**6**

* (Vrije University, Amsterdam, Netherlands)

Table 2.2 Drugs doses, concentration and molecular weight.

Drug	Dose	Molecular weight
Votucalis	mg/kg: 0.3, 1, 3, 10, 20, 40 µmol: 0.00032, 0.001, 0.0033, 0.011, 0.021, 0.043	**23.3KDa**
PF-0868087	mg/kg: 1, 3, 10 µmol: 0.072, 0.22, 0.72	**346.47 g/mol**
Mepyramine maleate	mg/kg: 10, 20 µmol: 0.622, 1.24	**401.46 g/mol**
Ranitidine hydrochloride	mg/kg: 15 µmol: 1.07	**350.86 g/mol**
JNJ 7777120	mg/kg: 20 µmol: 1.8	**277.75 g/mol**
Bavisant	mg/kg: 10 µmol: 0.59	**420.4 g/mol**
Immepip dihydrobromide	µg: 100 µmol: 0.305	**327.06 g/mol**
Compound 48/80	µg: 100 µmol: 0.159	**629.1 g/mol**
Chloroquine diphosphate	µg: 200 µmol: 0.388	**515.86 g/mol**

2.4 Itch model

2.4.1 Induction of scratching behaviour using histamine dependent and histamine-independent pruritogens

Mice were subjected to itch behaviour, induced by injection of pruritogens. Histamine-dependent itch was induced by compound 48/80 (100 µg; Cat. No. C2313; Sigma-Aldrich, Suffolk, United Kingdom) or histamine dihydrochloride (10 µmol; Cat. No. H7250-5G; Sigma-Aldrich, United Kingdom) and histamine-independent itch was induced by chloroquine (chloroquine diphosphate salt, 200 µg; Cat. No. C6628; Sigma-Aldrich, United Kingdom) as previously described by Sun et al., 2009 and Obara et al., 2015. Intracutaneous (i.d.) injection of compound 48/80 has been shown to directly affect sensory nerve fibres (Dunford et al., 2007) and stimulate release of endogenous histamine from cutaneous mast cells in a Ca^{2+}-dependent manner (Barrett et al., 1985; Tomoe et al., 1992), that results in a high incidence of scratching behaviour in the injected site by the hind paws, an itch-associated behaviour. This is used as an index for the study of itch not only in experimental animals but also in humans (Inagaki et al., 2002; Obara et al., 2015). In contrast, chloroquine is a Mas-related G protein-coupled receptor A3 (MrgprA3) agonist that upon intracutaneous administration elicits scratching behaviour resulting from activation of MrgprA3+ primary sensory neurons that were shown to be essential for itch sensation (Shiratori-Hayashi et al., 2019).

Itch-inducing agents (compound 48/80, chloroquine or histamine) were dissolved in sterile saline and a volume of 50 µL was injected subcutaneously (s.c.) or (i.d.) into the nape of the mouse neck. Before beginning the itch experiments, the back of the mouse neck was shaved with clippers (Oster, small animal clipper, model A5-00), and the tail was marked. Animals were given 30 minutes to acclimate to a small plastic chamber (12 × 10 × 10 cm).

For administration of pruritogens, mice were removed from the chamber, gently restrained, and injected with pruritogens. Itch behaviour was recorded with a digital video camera which was developed upon local administration of pruritogens and the observations are analysed following the experiment schedule as appeared in Figure 2.1. Control animals received 50 µL of equivalent vehicle solution. It is also important to note

that, at all times, mice were closely observed through all procedures for any abnormal changes in behaviour that might indicate discomfort, distress or of significant ill related to procedure or drug administration.

Behavioural assessment. In all experiments the observer was not aware of the doses and/or treatment administered. One scratch was defined as a "lifting of the hind limb toward the injection site (the shaved area of the neck) and then replacing the limb back to the floor, regardless of how many scratching strokes (bouts of scratching) took place between the lifting and replacing movements" (Obara et al., 2011; Shimada et al., 2006).

Mice were videotaped for 40-minutes immediately after receiving the injection, with no one else present in the recording room. Immediately following recording, the videotapes were re-played, and the number of scratching bouts directed toward the injection site was counted by an investigator who was blinded to the treatment. The scratching was counted as the total number of scratches that take place in 40-minutes observation period, or the cumulative scratches recorded for 40 minutes at 5 minutes intervals. Each mouse was used only once, in one experiment.

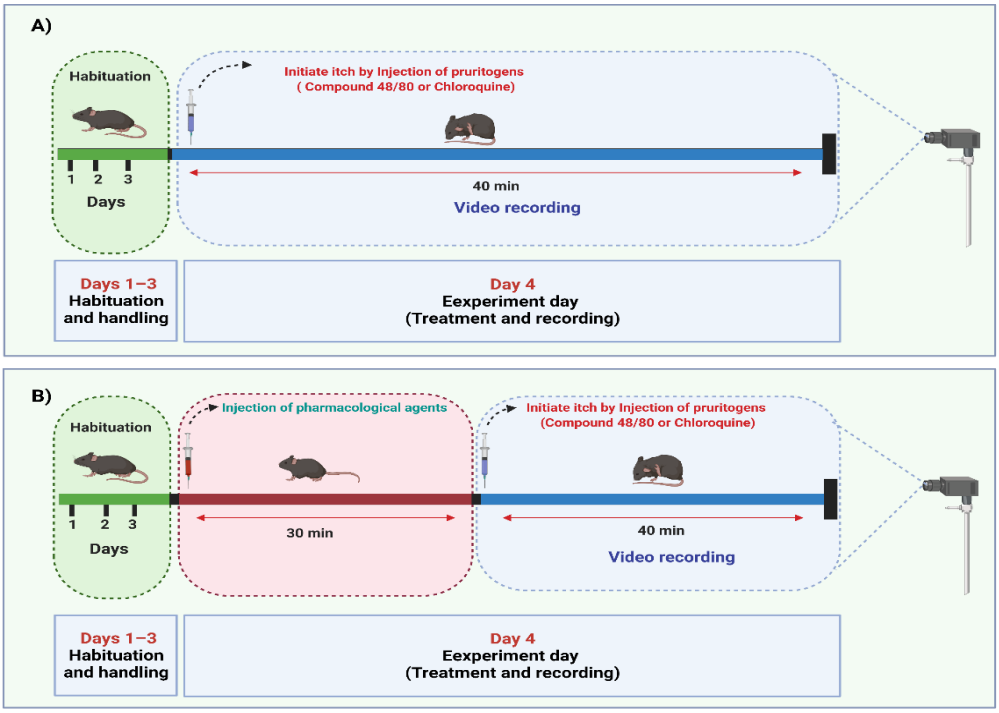


Figure 2.1 Schematic representation of the experimental timeline. The experiment lasted 4 days in total. **(A)** Before treatment with pharmacological agents. **(B)** After treatment with pharmacological agents. The (green line) represents the habituation days (30-minute) for 3 consecutive days, (red line) represents the time after injection of the pharmacological agent and (blue line) represent the recording time with a digital video camera after induction of itch .

2.4.2 Induction of scratching behaviour using H_4R agonist (*trans*-VUF16129)

In a separate group of animals, scratching behavior was induced by injecting of pruritogen selective H_4R agonist, *trans*-VUF16129 at the back of the neck, between the ears of the animal *via* intradermal (i.d.) route.

Firstly, itch-inducing agents were dissolved in sterile saline under red light and 50 µL (histamine or *trans*-VUF16129) was administered i.d. into the nape of the mouse neck which was shaved before starting itch experiments using clippers (Oster, small animal clipper, model A5-00), and the tail was marked. Animals were given 30 minutes to acclimate to a small plastic chamber (12 × 10 × 10 cm).

For administration of pruritogens, mice were taken out from the chamber, softly restrained, and injected with pruritogens. Itch behaviour in the mouse that developed after local administration of pruritogens was recorded with a digital video camera and analysed following the experiment schedule as appeared in Figure 2.2. Control animals received 50 µL of equivalent vehicle solution. It is also important to note that, at all times, mice were closely observed through all procedures for any abnormal changes in behaviour that might indicate discomfort, distress or of significant ill related to procedure or drug administration.

Mice were videotaped for 40-minutes immediately after receiving the injection, with no one else present in the recording room. Immediately following recording, the videotapes were re-played, and the number of scratching bouts directed toward the injection site was counted by an investigator who was blinded to the treatment. The scratching was quantified as the total number of scratches across a 40-minute observation period, or the cumulative number of scratches observed for 40 minutes at 5-minute intervals. Each mouse was used only once, in one experiment. Whole experiment was run under a red light as this protects chemical properties of *trans*-VUF16129.

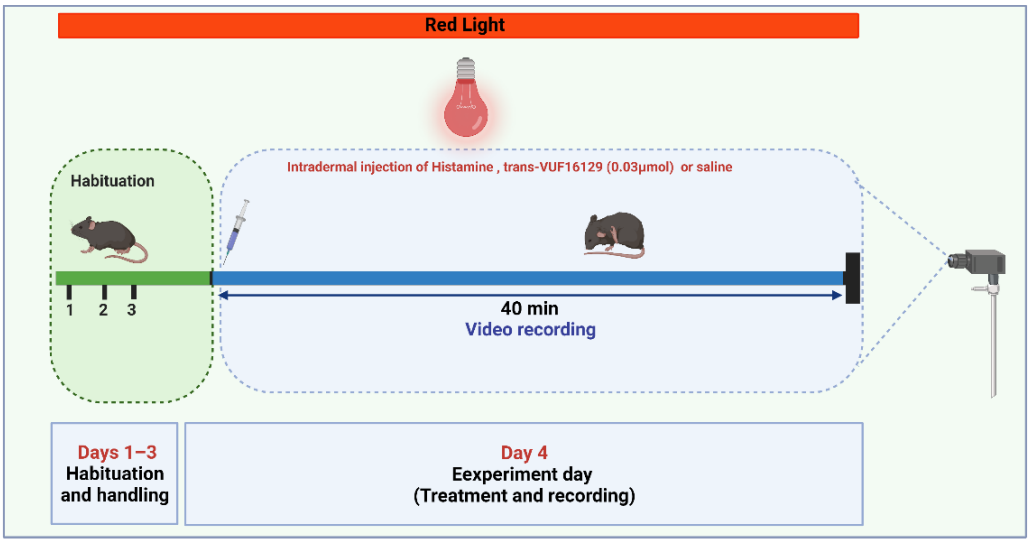


Figure 2.2 Schematic representation of the experimental timeline after use of photo-switchable ligands. The experiment lasted 4 days in total. The green line represents the habituation days (30-minute) for 3 consecutive days, and blue line represent the recording time with a digital video camera after induction of itch. Whole experiment was run under red light.

2.5 Neuropathic pain model

In view of the fact that neuropathic pain treatment is often poorly treated and many patients are deeply unsatisfied, *in vivo* and *in vitro* basic studies using animal models have been used to provide clues into the etiology and pathophysiology of the neuropathic pain as well as study the mechanism of action of many drugs and modify the available treatment protocols (Asante, 2009). One of the most intensively studied animal models for neuropathic pain are those involving a mechanical trauma to peripheral nerves such as model of the chronic constriction injury (CCI) of the sciatic nerve that results in the peripheral neuropathic pain development and was used in the work presented in this thesis (Bennett and Xie, 1988).

2.5.1 Chronic constriction injury (CCI) model

Mice were subjected to peripheral neuropathy induced by CCI similar to the approach described earlier by Bennett and Xie, 1988 and Obara et al., 2013. All surgical procedures were carried out under aseptic conditions in anesthetized animals. Under isoflurane inhalation, anaesthesia was delivered *via* a nose cone (1-3% isoflurane with oxygen as the carrier gas for maintenance; up to 5% for induction), always ensuring that the pinch reflex of the front limb was stopped and assessing the respiratory rate before starting the surgery. Loss of body temperature was prevented by placing the animal on an

automated heating pad (37.5 ± 0.5 °C). On the lateral surface of the left thigh, the skin was shaved then an incision was made parallel to the sciatic nerve, just below the left hipbone. The right sciatic nerve was exposed upon separating the biceps femoris and the gluteus superficialis and CCI injury was made by tying three loose ligatures (4.0 silk) around the exposed sciatic nerve as illustrated in Figure 2.3. The ligatures were tied with a 1 mm spacing until a brief twitch in the respective hindlimb was elicited. The muscles and skin were then closed with a 5.0 silk suture. After surgery, the mice were returned to their home cages in holding area and monitored closely for 2-4 hours. The animal wellbeing and surgical wounds were observed and evaluated at least once a day for next 7 days. The animal's weight was also monitored and recorded post-operatively and compared against its preoperative weight. In the sham-operated control mice, the left sciatic nerve was isolated and exposed without ligation. The muscles and skin were then closed with a 5.0 silk suture. Both mechanical threshold and thermal sensitivity were measured before nerve injury treated as basal pain threshold and then mice were tested after day 7 of surgery and sustained for 11 days.

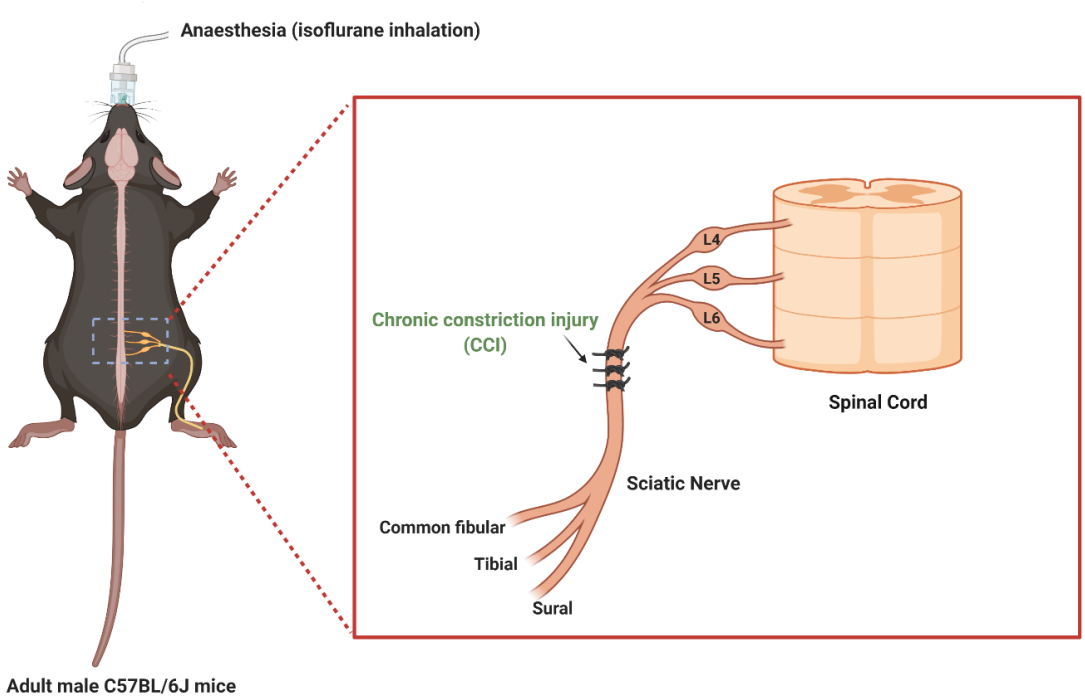


Figure 2.3 Schematic diagram showing sciatic nerve position and ligature placement in the chronic constriction injury. three ligatures were loosely applied around the sciatic nerve at approximately 1mm intervals. The information in the figure is based on Bennett and Xie, 1988; Obara et al., 2013 and Yalcin et al., 2014.

2.6 Assessment of neuropathic pain (behavioural testing)

In this study the behavioural signs of the animals represent two different components of neuropathic pain: mechanical allodynia and thermal hyperalgesia. Before surgery mice were subjected to behavioural testing to establish a baseline to compare with the post-surgical values, with values for the uninjured contralateral and injured ipsilateral paw measured from day 3 following CCI. In all experiments, mice were habituated to a plexiglas behaviour chamber for 2-3 days before the beginning of the experiment. The experimenter remained blind to the treatment during the testing procedure. Behavioural testing was carried out to assess the development and progression of chronic pain with a focus on mechanical allodynia and thermal hyperalgesia. Animals that displayed injury induced behavioural reflex sensitisation were used for further pharmacological or biochemical studies, in order to explore the underlying mechanisms responsible for developing these chronic pain states, which is vital for the development of new effective analgesics.

2.6.1 General observations

Animal were observed on a regular basis to assess the animal's wellbeing posture, gait and condition of the injured hind limb and normal behaviour (for example activity, feeding and grooming behaviours). Following the licence, any animal displaying signs of autotomy (rarely observed), excessive distress and ill health (for example excessive facial grimacing, decreased grooming behaviour, biting, or gnawing of the injured paw or leg, ataxia), would be euthanized by Schedule 1 after consultation with NVS. All animals used in this study showed, normal behaviour and maintained normal weight following surgery and during all testing.

2.6.2 Mechanical allodynia

To assess mechanical allodynia each animal was placed on a metal mesh floor allowing the experimenter to reach the plantar surface of the hind paw from beneath, unobserved by the animal (Figure2.4). Mechanical sensitivity was assessed in mice by measuring the withdrawal threshold of the paw ipsilateral to the site of injury in response to mechanical stimuli using von Frey filaments (Stoelting, Wood Dale, IL, USA).

During testing, mice were kept in plastic cubicle cages measuring (10 × 6 × 6 cm) having a metal mesh floor. Then, mice were given time for 10-15 minutes to habituate before testing. Calibrated nylon filaments were applied in ascending order, and perpendicular to the midplantar surface of the hind paw for 2-3 seconds and repeated three times (Obara *et al.*, 2003; Chaplan *et al.*, 1994; Bourquin *et al.*, 2006). The threshold was taken as the lowest force that induced rapid response of paw withdrawal (Obara *et al.*, 2011). The strength of the von Frey stimuli used in the present experiments ranged from 0.04 to 2.0 g.

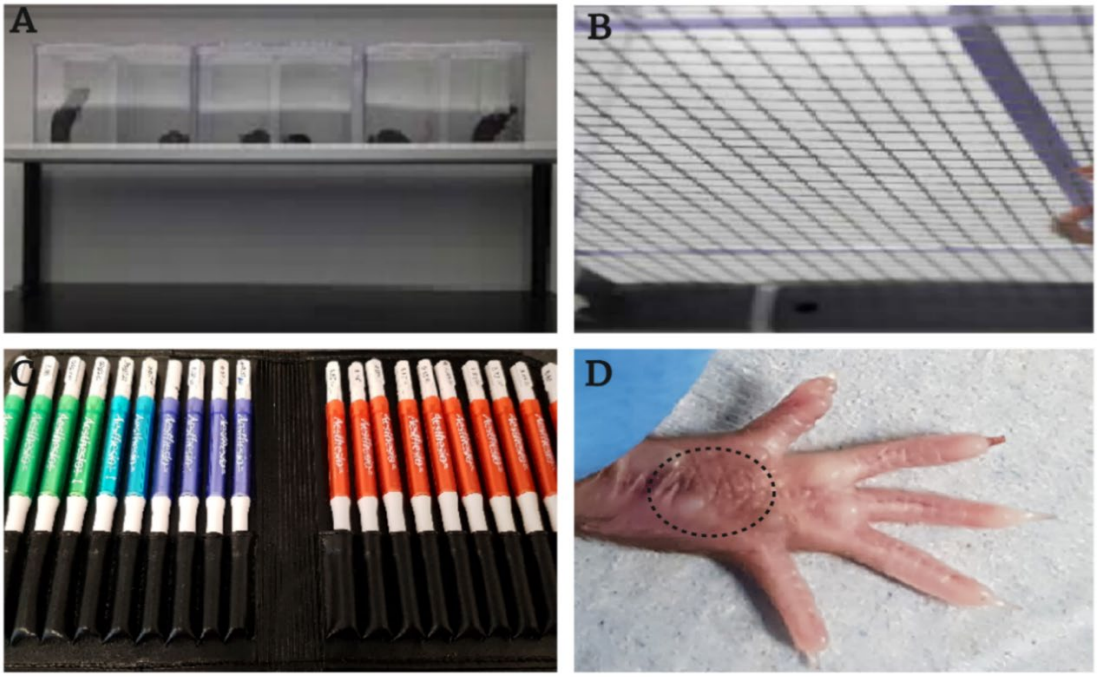


Figure 2.4 von Frey test. (A) Mice were placed individually in plexiglass cages on an elevated mesh platform. **(B)** Mesh platform: open grid of square holes (5 × 5 mm) for paw access. **(C)** Set of monofilaments made of nylon calibrated ranged from 0.04 g to 2.0 g. **(D)** Plantar view, mouse hindlimb paw. The dashed area in the image corresponds to tested area with the von Frey hair. (adapted from Alsubaiyel, 2019).

2.6.3 Thermal hyperalgesia

Thermal hyperalgesia was assessed using the Hargreaves test (Model 400 Heated Base from IITC Inc, Ca, USA) as described earlier (Hargreaves *et al.*, 1988). Using Hargreaves' thermal device and applying radiant heat (30-55°C) to the mid-plantar glabrous surface of the hind paw, thermal hyperalgesia was monitored as shown in Figure 2.5. Mice were gently placed in plastic cubicle cages measuring (12 cm high: 10 × 10 cm) with a glass

floor (1.2 cm thick) at room temperature. Mice were first given time about (10-15 minutes) to habituate prior to testing. A heat source was positioned under the glass floor of the cage, directly beneath the hind paw being tested. A digital timer automatically recorded the duration between the start of the stimulus and the withdrawal of the paw from the heat source. To gain an average baseline paw withdrawal latency, the intensity of the thermal stimulus was adjusted to approximately 9-11 seconds. A maximum time of 20 seconds was set on the timer, to avoid unnecessary tissue damage. The test was repeated in triplicate on the same mice, with a 1minute minimum resting time between each measurement (alternating between paws) to avoid any sensitization of the paw.

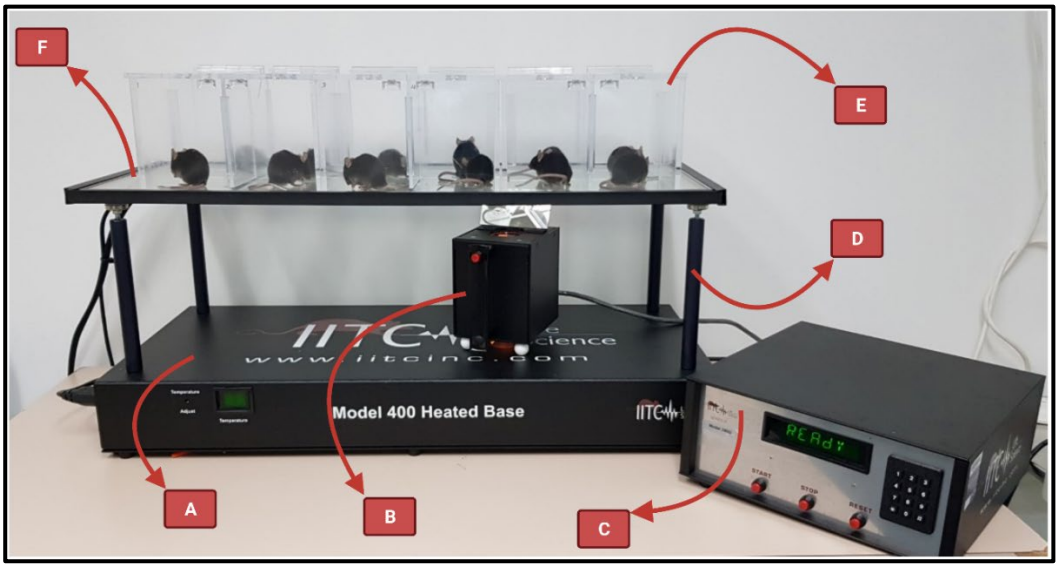


Figure 2.5 Hargreaves test. (A) large platform, **(B)** Emitter (detector vessel), **(C)** Controller for manipulation of test settings such as infrared intensity, display of reaction time, and power input port, **(D)** Supporting column, **(E)** Animal enclosure (holds up to 12 mice at a time) and **(F)** Framed glass panel for optimal thermal conductance the glass panel was kept clean and protected from damage.

2.6.4 Experimental timeline of neuropathic pain study

A diagram of the experimental design is shown in Figure 2.6. Baseline for mechanical and thermal stimulation thresholds was taken one day (day -1) before the induction of neuropathic pain by CCI (day 0). On day 0, CCI was performed (see section 2.1.1). Following recovery, another post-surgery baseline was taken on day 6 and 7 to assess the development of neuropathic pain in response to mechanical and thermal stimuli. After that, on the 7th day post CCI the pharmacological agents or equivalent vehicle solutions without the drug, were administered according to its respective groups. The

blue line represent behavioural test measured in response to mechanical stimuli using von Frey filament and the green line represent behavioural test measured in response to thermal stimuli using Hargreaves test. Data for both behavioural tests were recorded 30 min after the administration of the final treatment. More specific details including materials, drug dosage, rout of administrations was described in the methods section of the relevant chapters.

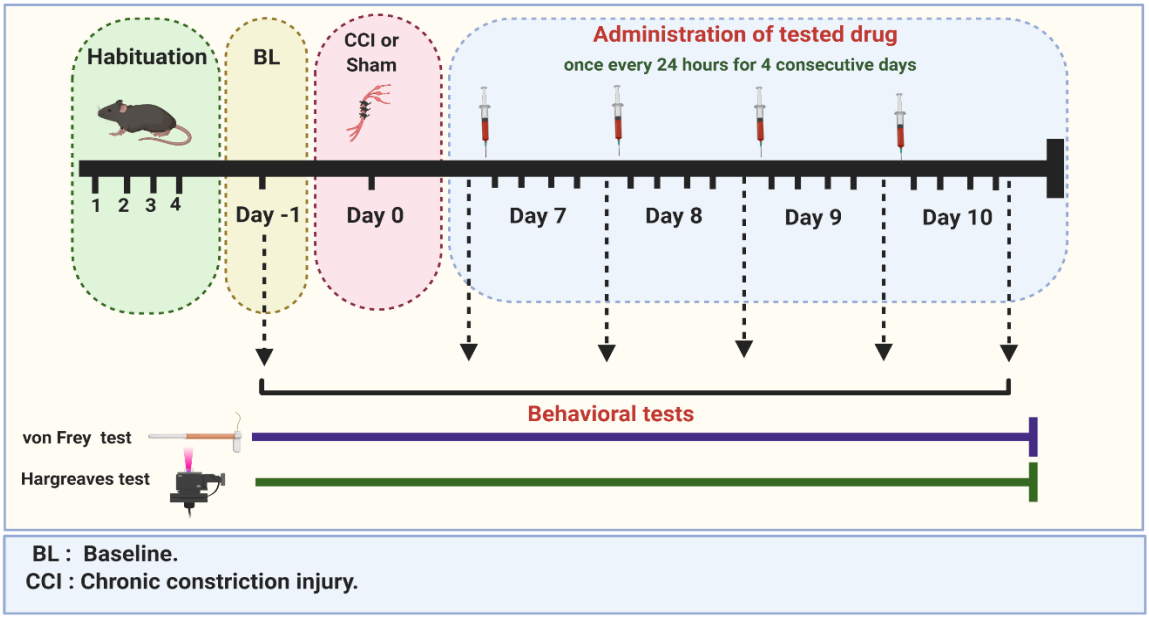


Figure 2.6 A schematic diagram summering a timeline of the experiment. Baseline (day -1) for mechanical and thermal stimulation thresholds was taken one day before the induction of neuropathic pain by chronic constriction injury (CCI) and Sham. Following recovery, another post-surgery baseline was taken on day 6 and 7.After that, on day 7 post-CCI the tested drugs or equivalent vehicle solutions without the drugs, were administered on day 7-10 once daily.

2.7 Biochemical assays

The purpose of these experiments was to evaluate the changes in the expression of different types of protein that could be defected in both ipsilateral and contralateral sides of the spinal cord and sciatic nerve and that may occur after nerve injury to the sciatic nerve. This analysis could help us to make comparisons between these sites so that similarities and/or differences could be analysed. Tissue was taken from the spinal cord and sciatic nerves in both neuropathic and sham-control animals. These sample were examined for changes in protein levels due to nerve injury by immunoblotting method both ipsi and contralateral in CCI and sham control groups.

2.6.1 Immunoblotting (Western blotting)

Western blotting is the alternative name for immunoblotting. It is an extensively used and widely accepted analytical method to identify and quantify the expression of a specific protein of interest from the tissue homogenate or a cell sample (Kurien and Scofield, 2006). Western blotting was performed as described earlier (Chen et al., 2015; Obara et al., 2013). The mechanism of this method involves gel electrophoresis, where there is the separation of proteins based on their molecular weights (Figure 2.7). Then, the proteins were transferred to specific Polyvinylidene fluoride (PVDF) membrane and were allowed to produce a band for each peptide or protein using a specific antibody.

2.7.1.1 Tissue preparation

In the last day of the treatment mice were sacrificed by using cervical dislocation according to Schedule 1. In both group of animals shams and CCI mice, spinal cord and sciatic nerves were collected. The spinal cord section was transacted at the level of the lumbar area (L4-L6). Subsequently, this part of the spinal cord was removed and cut in half along its length allowing the separation of ipsilateral and contralateral sides. The ipsi- and contra-lateral dorsal horn quadrants (L4-L6) were dissected out and immediately kept in dry ice. Until further processing, all tissues were stored at -80°C.

For protein extraction, each sample was homogenised in 150 µl lysis buffer (1% Np-40, 20 mM Hepes pH7.4, 100 mM NaCl, 100 mM NaF, 1 mM Na3VO4, 5 mM EDTA with 1× protease inhibitor cocktail (SIGMA); 1× phosphatase inhibitor cocktail I and II (Sigma-Aldrich) using a Precellys 24 Tissue Homogenizer (Life Science Centre, NCL, UK), and

incubated on ice for 120-minute to solubilize proteins then the homogenates were centrifuged at 21,000 rpm for 15-minute at 4°C to precipitate the membrane and tissue debris. The supernatant that contains most of the soluble proteins was collected in sterile Eppendorf's tubes and stored at -20°C and the pellet was discarded.

2.7.1.2 Determination of total protein concentration for Western blotting

Following the manufacturer's instructions, quantify the total protein concentration in a sample by using a Bradford assay. Initially, 96-well microplates were loaded with 10µl of six known standard concentration (0 µM, 0.25 µM, 0.5 µM, 1 µM, 1.5 µM, 2 µM) and 2 µL of homogenized samples. All standards and samples run in duplicate and at the same time. 200µl of Bradford assay reagent (Bio-Rad, UK) was added to each of the wells containing the protein sample, and this was incubated for 30 minutes at 37^{o}C. After 30 minutes, the plate was put into a microplate spectrophotometer (MultiSkan GO, Thermo Scientific, Finland) and read at a wavelength of (λ= 562 nm). The data were used to construct of a calibration graph by plotting the absorbance of the standards against their concentrations. The calibration graph was used to calculate or interpolate the protein concentrations of the samples.

2.6.1.3 Procedure for loading and running the gel

After finding the total protein concentration, tissue extracts with 15-30 µg of proteins per sample and the loading buffer solution (Sample Buffer, Laemmli 2x; Cat. No. S3401, (Sigma-Aldrich) were mixed. The sample was boiled at 95°C for 5 min and after that a volume of ~15µl per well of the sample was run on 4-20% Criterion TGX Stain-Free Precast gels (Bio Rad, Hemel Hempstead, UK) in Tris/Glycine/SDS running buffer (25Mm Tris, 192Mm Glycine, 0.1% (w/v) SDS, Ph8.3) (Bio-Rad, Hemel Hempstead, UK) at 200 V for 30 min using the Bio-Rad PowerPac system. Then, proteins were electrophoretically transferred onto a PVDF membrane (Bio-Rad Hemel Hempstead, UK) using the Bio-Rad Trans-Blot Turbo Transfer system for this, the gel and membrane were assembled into a sandwich as shown in step number 2 in Figure 2.7, then Turbo Transfer system was run at 2.5 ampere (A) and 25 (V) for 7 minutes. To prevent the nonspecific binding of the antibodies, at room temperature all the membranes were then blocked for 60 minutes using either 4% non-fat dry milk solution in TBS-Tween 20 or in 10 mM Tris-HCl

pH 5 7.5, 150 mM NaCl, 0.05% Tween 20 (Sigma) with 0.24% I-Block (Applied Biosystems, Paisley, UK). Then, the blot was probed with a primary antibody at 4°C for overnight to 48 hours.

2.6.1.4 Antibody labelling

Different primary antibodies were applied to specifically bind the protein of interest as listed in Table 2.2. After incubation with the antibody, membranes were then washed in phosphate-buffered saline (PBS) 3 x 10 min at room temperature and then incubated in host-dependent secondary antibody (anti-mouse or anti-rabbit) horseradish peroxidase (HRP) (goat anti-mouse IgG (H+L)-HRP conjugate; Cat. No. 170-6516, Bio Rad, USA) and (Goat anti-rabbit IgG (H+L)-HRP conjugate; Cat. No. 170-6515, Bio Rad, USA) for 60-minute at room temperature. Secondary antibodies were used at dilution of 1:1000 in either 0.24% I-block or 4% non-fat milk. Subsequently, membranes were washed (PBS) 3 x 10 minutes at room temperature. After visualization, membranes were then washed and incubated with GAPDH antibody overnight at 4˚C and further processed as described above.

2.6.1.5 Visualisation of antibody binding

To detect proteins of interest, HRP activity was visualized upon application of Bio-Rad Clarity Western ECL substrate (Clarity western lumiol/enhancer solution and peroxide solution at a ratio of 1:1, Hemel Hempstead, UK) and using ChemiDoc MP (Biorad Hemel Hempstead, UK) imaging system. Densitometry was performed using Image J to estimate protein expression on each membrane. The ratio of phosphorylated/total protein was calculated and normalized with the intensity of the GAPDH signal.

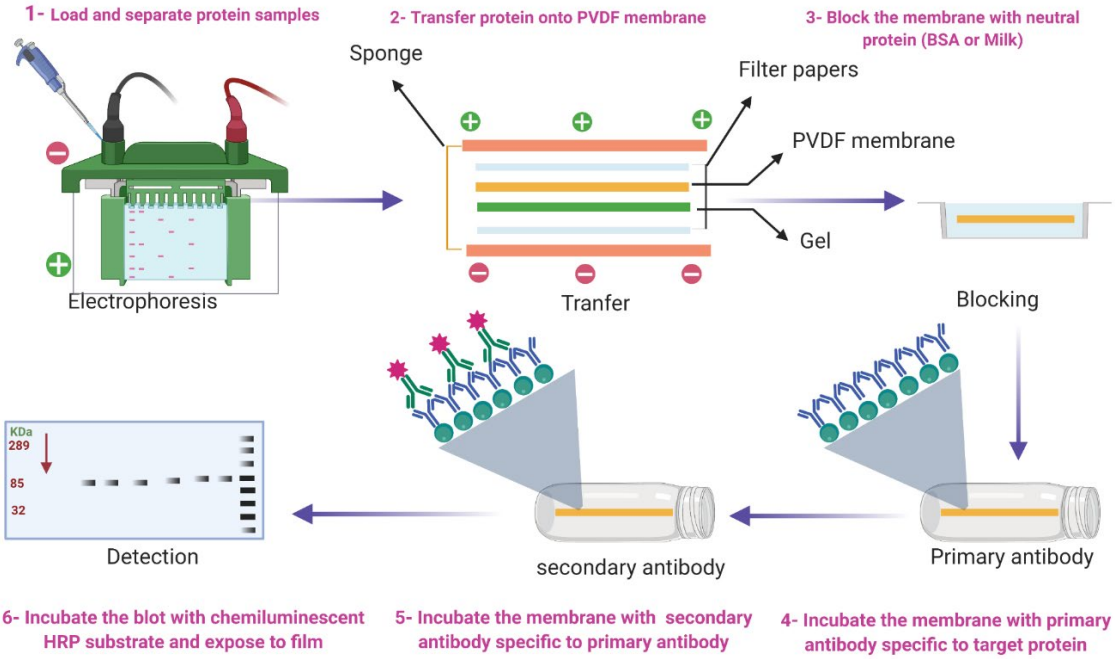


Figure 2.7 Schematic of the step-by-step process of Western Blot Procedure used in this thesis. 1- interested proteins are loaded into the wells are separated by using gel electrophoresis usually SDS-PAGE. **2-**The proteins are transferred to a sheet of special blotting paper called PVDF membrane.**3-** The blot membrane is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the nitrocellulose.**4-** primary antibody is then added to the solution, which can bind to its specific protein (48 h 4°C). **5-** Incubate the PVDF membrane with secondary antibody specific to primary antibody for 1 hour at room temperature, then dd electrochemiluminescence (ECL) solution and left in the dark for 5 minutes. **6-** using ChemiDoc MP imaging system to detect proteins of interest. The information in the figure is based on Kurien and Scofield, 2006; Liu et al., 2014 and Mishra et al., 2017.

Table 2.2 Western blotting antibodies used in the experiments.

Protein targeted	Host	Bands molecular weight (kDa)	Primary antibodies dilution & incubation	Secondary antibody	Secondary antibodies dilution & incubation	Primary antibody Supplier
mTOR	Rabbit polyclonal	**289**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
P70 S6 Kinase	Rabbit polyclonal	**70, 85**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
S6 Ribosomal Protein	Rabbit monoclonal	**32**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
4E-BP1	Rabbit monoclonal	**15 to 20**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
GAPDH	Mouse monoclonal	**38**	1 in 2000 24 h 4°C	Goat anti-mouse	1 in 1000 1h 25°C	**EMD Millipore** (Burlington, MA, USA)
HRH3	Rabbit monoclonal	**49**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Abcam** (United Kingdom)
GluR1	Rabbit polyclonal	**102**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Abcam** (United Kingdom)
Phospho-mTOR	Rabbit polyclonal	**289**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
Phospho-P70 S6 Kinase	Rabbit polyclonal	**70, 85**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
Phospho-S6 Ribosomal Protein	Rabbit polyclonal	**32**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
Phospho-4E-BP1	Rabbit monoclonal	**15 to 20**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Cell signalling Technology** (Beverly, MA, USA)
Phospho-GluR1	Rabbit monoclonal	**102**	1 in 500 48 h 4°C	Goat anti-rabbit	1 in 3000 1h 25°C	**Abcam** (United Kingdom)

CHAPTER 3

Effect of systemic and local peripheral administration of a novel histamine-binding protein Votucalis on histaminergic itch

3.1 Introduction

Histamine is one of the most important neurotransmitters known to be involved in several physiological and pathological conditions, including insomnia (Krystal et al., 2013), chronic neuropathic pain (Obara et al., 2020), inflammation (MacGlashan, 2003), allergy (Thurmond et al., 2008), motion sickness (Takeda et al., 1986) and importantly, itch (Baron et al., 2001). It is synthesised, stored and released from neuronal and non-neuronal cells, such as enterochromaffin-like cells, mast cells and basophils (Borriello et al., 2017; Best et al., 2017; Prinz et al., 1999). Histamine exerts its effects *via* a family of G protein-coupled receptors, known as the HRs. Currently, there are four recognised histamine receptors called as H_1R, H_2R, H_3R, and H_4R. These receptors are expressed in both the CNS and PNS, and they exert an excitatory or inhibitory effects depending on the HR subtype (Panula et al., 2015; Lindskog, 2017). Depending on its concentration, site of action, and type of receptors implicated, histamine can both reduce and aggravate sensory perception of itch (Ikoma et al., 2006). Therefore, histamine and receptors have long been attractive targets for therapeutic interferences, especially in conditions where itching is a symptom requiring treatment and management (O'Donoghue and Tharp, 2005; Obara et al., 2020).

While histamine has been identified as a major mediator of itch in humans and rodents (Shim and Oh, 2008; Schmelz et al., 1997), the mechanism by which histamine induces itch is not fully understood (Rossbach et al., 2011). In general, using selective receptor agonists and antagonists is one of the important tools to study the mechanisms and pharmacology of the receptors involved in itch (Van Der Goot and Timmerman, 2000). Indeed, stimulation or inhibition of HR subtypes by using agonist or antagonist has led to a remarkable increase in the understanding the significance of histamine and its receptors in the mechanisms of itch (Van Der Goot and Timmerman, 2000). Furthermore, research has shown that when histamine is administered locally, the itch

is provoked in a dose-dependent manner (Keele and Armstrong, 1964). Also, compound 48/80, which causes mast-cell degranulation and local histamine release, was shown to produce itch in both humans and mice (Fjellner and Hägermark, 1981; Kuraishi et al., 1995).

The discovery of histamine-binding proteins within the saliva of arthropods ticks has yielded a potentially powerful research tool to re-examine the role of histamine in the regulation of itch (Chmelař et al., 2019; Weston-Davies et al., 2005). Ticks are a type of blood-feeding arthropods that suck blood from humans and animal hosts and have adopted a special and unique strategy to control host-defense mechanisms at the feeding site (Paesen et al., 1999; Ryffel et al., 2005). The tick's saliva contains a family of a bioactive mixture including protein and lipid with anti-inflammatory, anti-coagulant, anti-platelet, anti-fibrotic, anti-haemostatics, and immunomodulatory effects (Chmelar et al., 2012; Boulanger, 2018; Štibrániová et al., 2019). Despite of the fact that a large number of bioactive tick salivary molecules have been discovered, their examination for medical purposes remains in its early stages. Pre-clinical and clinical studies have been done only on a small number of tick salivary compounds, mainly proteins (Aounallah et al., 2020).

Votucalis (also known as rEV131 or HPB1) is one of the proteins extracted from female tick *Rhipicephalus appendiculatus*, with histamine binding activity or in other words, with histamine scavenger activity (Paesen et al., 1999). This histamine binding protein is linked to a broad ligand-binding protein family known as lipocalins. Votucalis is a histacalin, which binds histamine at two different internal binding sites with the high-affinity binding site (as shown in Figure 3.1) displaying a 100-fold higher affinity than H_1R and H_2R and a similar affinity to the H_3R and H_4R (Paesen et al., 1999; Paesen et al., 2000; Nuttall and Labuda, 2004; Ryffel et al., 2005). Interestingly, both pre-clinical and clinical studies show therapeutic effects reached by scavenging endogenous histamine *via* recombinant Votucalis. These studies focused on pathological conditions where histamine is recognized as a key player, such as acute respiratory distress syndrome (ARDS), asthma and conjunctivitis and allergic rhinitis (Ryffel et al., 2005; Weston-Davies et al., 2005).

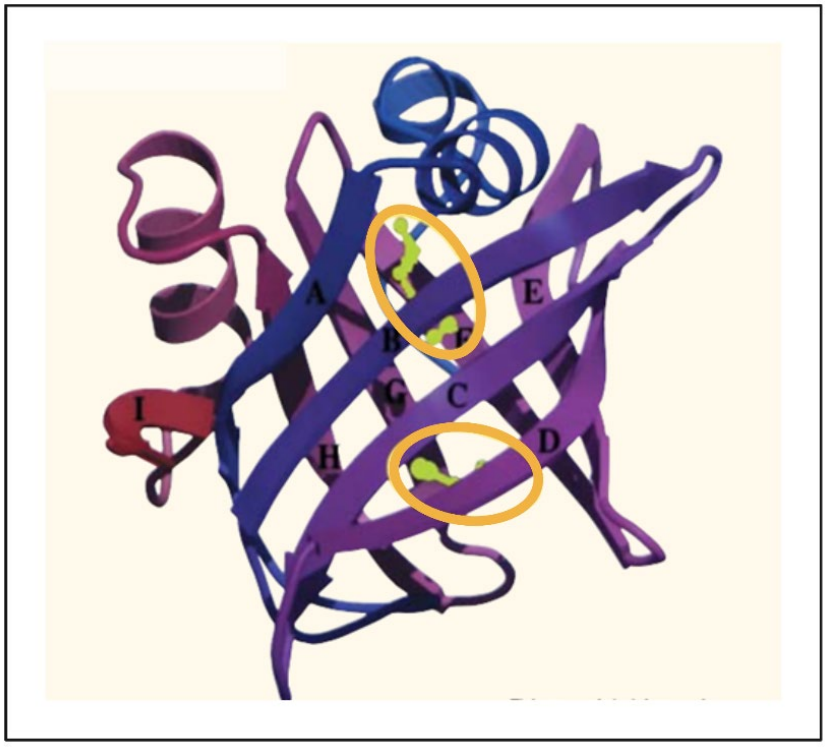


Figure 3.1 Votucalis structure and histamine binding sites. Histamine occupying at two high affinity binding sites (indicated in orange) (adapted from Annual Report Evolutec Group 2004; Macilwain, 2006)

This study aimed to further emphasizes the importance of histamine in the controlling of acute itch based on scavenging of endogenous histamine by Votucalis and focused on mouse models of acute itch. We hypothesised that selective targeting of peripheral histamine that stimulates itch response by Votucalis would result in anti-pruritic effects that may suggest novel and potentially improved therapeutic strategy in the regulation and control of this condition.

3.2 Materials and Methods

3.2.1 Subjects

Experiments in this chapter were carried out using adult male C57BL/6J mice (8 weeks of age; 20–25 g; Charles River Laboratories, Harlow, UK) as previously described in Chapter two (Section 2.2). The itch-inducing procedure, behavioural assessment and measurement of scratching in this Chapter were as previously described in chapter two (Section 2.4.1), unless otherwise stated. A schematic diagram of this experiment is shown in Figure 3.2.

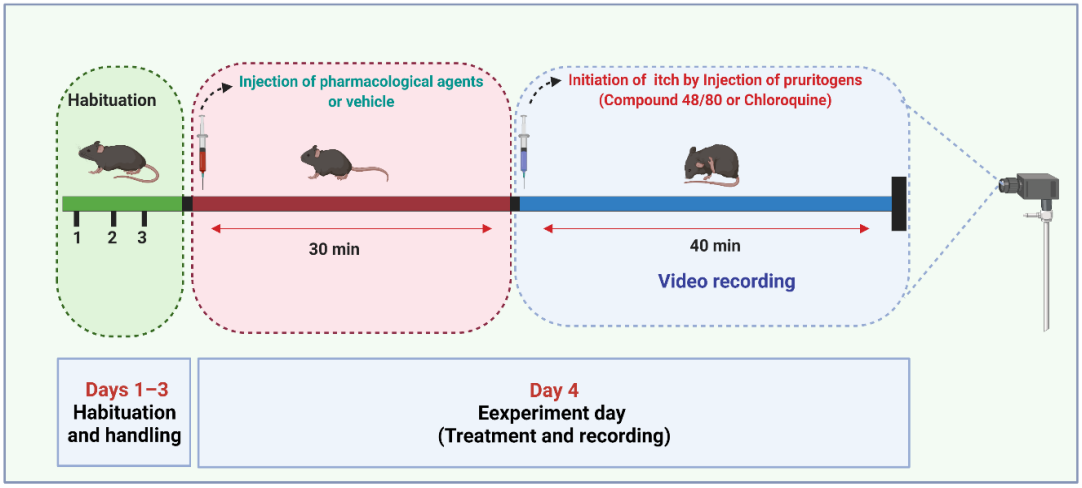


Figure 3.2 Schematic representation of the experimental timeline. The experiment lasted 4 days in total. The green line represents the habituation days (30 minutes) for 3 consecutive days, the red line represents the time after injection of the pharmacological agent and the blue line represent the recording time with a digital video camera after induction of itch.

3.2.2 Preparation and administration of drugs

Votucalis was provided by Akari Therapeutics Plc (UK) as a stock solution of 5.8 mg/mL in phosphate-buffered saline and was stored at -80°C. For all administrations, Votucalis was defrosted immediately before injections and prepared in a vehicle (sterile saline; 0.9% NaCl; Fresenius Kabi Ltd., UK) solution at required concentrations as described below. The isolation, cloning and detailed crystal structure of Votucalis were reported previously (Paesen et al., 1999; Paesen et al., 2000).

Systemic intraperitoneal (i.p.) administration. Mice were weighed and randomized to receive either Votucalis or vehicle (saline). They were injected i.p. with Votucalis at 1, 3, 10, 20 and 40 mg per kg body weight or vehicle (saline) solution without Votucalis as a control group. Votucalis/vehicle was injected 30 minutes before injection of pruritogens.

Local peripheral subcutaneous (s.c.) administration. Mice were weighed and randomized to receive either Votucalis or vehicle (saline). They were injected s.c. with Votucalis at 0.3, 1, 3, 10 and 20 mg per kg body weight or vehicle (saline) solution without Votucalis as a control group. Votucalis/vehicle was injected once 30 minutes before injection of pruritogens.

To determine the role of histamine receptors on the anti-pruritic effects produced by Votucalis, selective H_1R antagonist (mepyramine maleate, 10 and 20 mg/kg; Sigma-Aldrich, UK), selective H_2R antagonist (ranitidine hydrochloride, 15 mg/kg; Tocris Bioscience, UK) and selective H_4R antagonist (JNJ 7777120, 20 mg/kg; Tocris Bioscience, UK) were co-injected with Votucalis or alone 30 minutes before injection of pruritogens. The antagonists were dissolved immediately before injections (i.p. or s.c.) in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK), with the exception of JNJ 7777120 which was dissolved in DMSO (dimethyl sulfoxide, 5%; Sigma-Aldrich, UK). Control animals received equivalent vehicle (saline or 5% DMSO) injections. All drugs were injected in a volume of 100µl (i.p.) and 50µl (s.c.). The concentration, doses and timing of injections (antagonists) were made depending upon previously published reports (Bell et al., 2004; Dunford et al., 2007; Rossbach et al., 2011).

3.2.3 Itch model

4.2.3.1 Induction of itch and behavioural assessment

The method for the induction, recording and assessment of scratching behaviour is described in detail in Chapter 2.

3.2.4 Design of the experiments

3.2.4.1 To prove the anti-pruritic efficacy of Votucalis after systemic and local peripheral administration two experiment were conducted

Experiment 1: Anti-pruritic efficacy of systemic administration of Votucalis

After 3 consecutive days of habituation mice were randomly assigned into 12 groups as indicated in Table 3.1. On the experimental day (4th day), mice were weighed and habituated to a small plastic chamber for 30 minutes. Then, each mouse received a different dose of Votucalis systemically (1-40 mg/kg, i.p.) in volume 100µl 30 min before histamine dependent (compound 48/80, 100µg), or histamine-independent (chloroquine, 200µg) pruritogen. Behavioural responses after Votucalis or vehicle were recorded for 40 minutes and analysed at 5-min intervals. Control animals received 100µL of equivalent vehicle solution without Votucalis**.** Figure 3.1 shows the experimental timeline and Table 3.1. shows a summary of doses, sites of injection and number of animals in each experimental group.

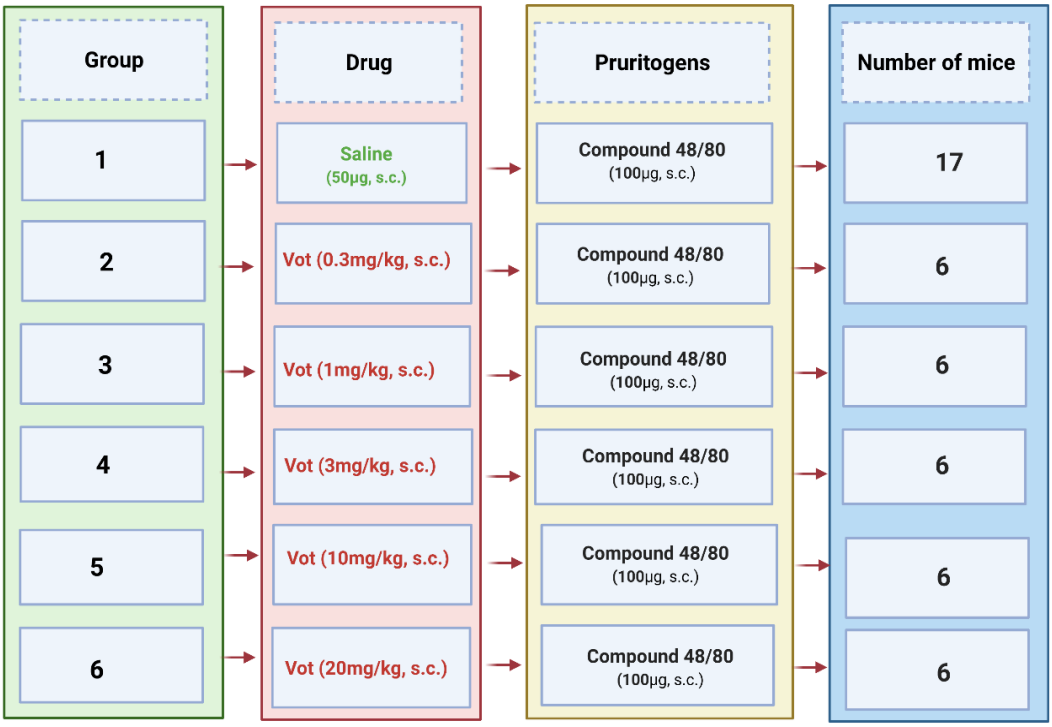
Table 3.1 Experimental groups in experiment 1

Mice were randomly assigned into 12 groups and received different doses of i.p. injection of Votucalis or vehicle 30 minutes after s.c. injection of compound 48/80 or chloroquine. **(Group 1)** Control group mice received i.p. injection of vehicle (saline) in a volume of 100 µL; **(Groups 2-6)** Different doses of systemic i.p. of Votucalis (1,3,10,20, and 40mg/kg) 30 minutes prior to s.c. injection of compound 48/80; **(Group 7)** Control group mice received i.p. injection of vehicle (saline) in a volume of 100 µL ; **(Groups 8-12)** Different doses of systemic i.p. of Votucalis (1,3,10,20, and 40mg/kg) 30 minutes prior to s.c. injection of chloroquine 200 µg.

Experiment 2: Anti-pruritic efficacy of peripheral administration of Votucalis

After 3 consecutive days of habituation, mice were randomly assigned into 6 groups as indicated in Table 3.2. On the experimental day (4th day), mice were weighed and habituated to a small plastic chamber for 30 minutes. Then, each mouse received a different dose of Votucalis locally (0.3-20mg/kg, s.c.) in volume 50µl 30 minutes before histamine-dependent (compound 48/80, 100µg) pruritogen. Behavioural responses after Votucalis or vehicle were recorded for 40 min and analysed at 5-min intervals. Control animals received 50 µL of equivalent vehicle solution without Votucalis. Figure 3.1. shows the experimental timeline and Table 3.2 shows a summary of doses, sites of injection and number of animals in each experimental group.

Table 3.2 Experimental groups in experiment 2



Mice were randomly assigned into 6 groups and received different doses of s.c. injection of Votucalis or vehicle. **(Group 1)** Control group mice received s.c. injection of vehicle in a volume of 50 µL. **(Groups 2-6)** Mice received s.c. injection of different doses of Votucalis (0.3- 20mg/kg) in a volume of 50 µL. Votucalis or vehicle was injected 30 minutes before injection of pruritogens

3.2.4.2 To determine the role of H_1R, H_2R and H_4R in histamine-mediated scratching three experiment were conducted:

Experiment 1: Role of H_1R

After 3 consecutive days of habituation, mice were randomly assigned into 5 groups as indicated in Table 3.3. On the experimental day (4^{th} day), mice were weighed and habituated to a small plastic chamber for 30 minutes. Then, each mouse was administered with a different dose of mepyramine, a selective H_1R antagonist (10 and 20 mg/kg, i.p., or 10 mg/kg, s.c.), 30 minutes before pruritogens to determine systemic and local peripheral effects of H_1R receptor antagonists on itch. Behavioural responses after mepyramine or vehicle were recorded for 40 min and analysed at 5-min intervals. Control animals received equivalent vehicle (saline) solution without mepyramine. Figure 3.3 shows the experimental timeline and Table 3.3 shows a summary of doses, sites of injection and number of animals in each experimental group.

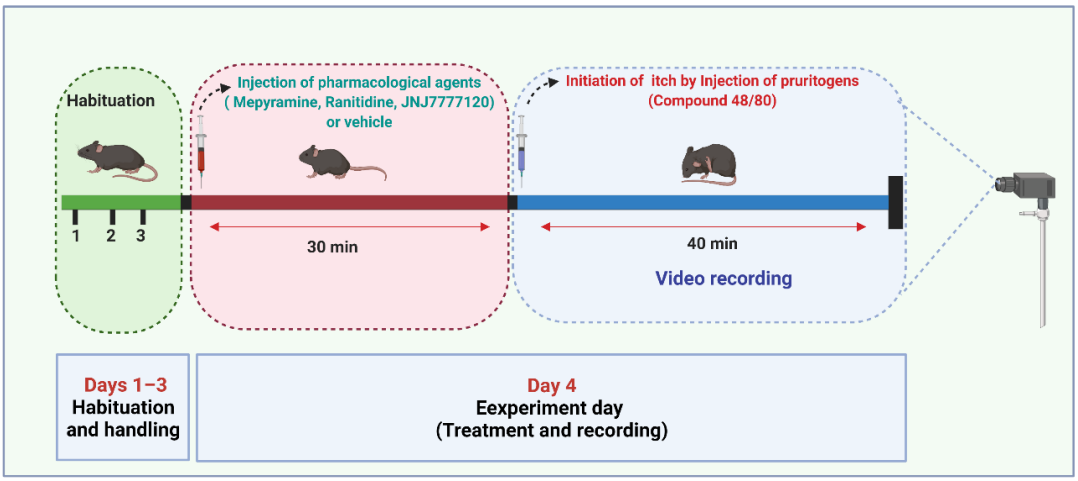
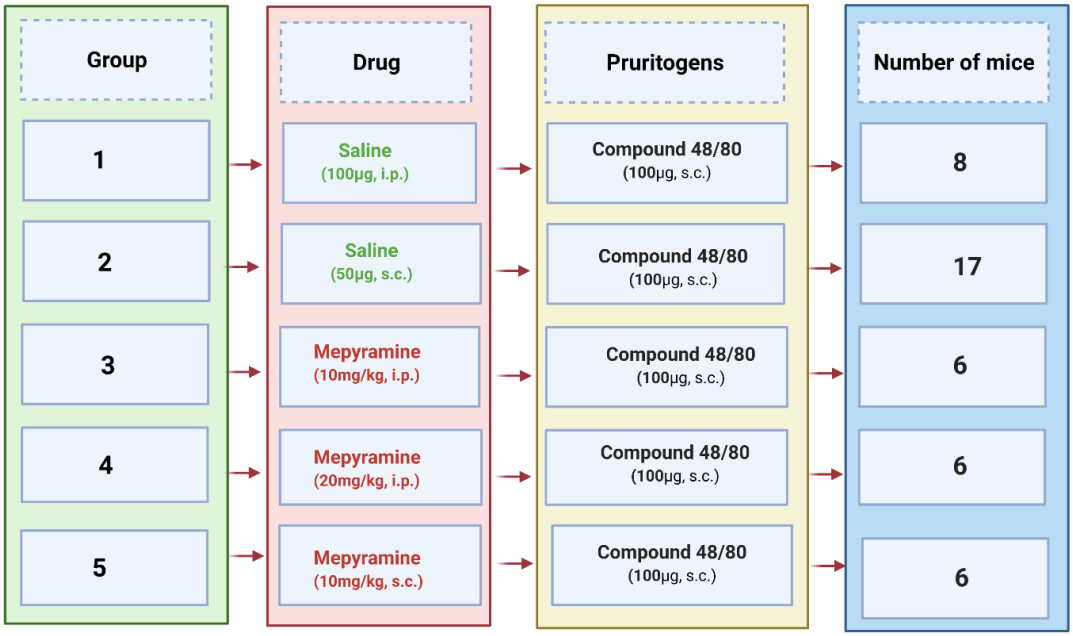


Figure 3.3 Schematic representation of the experimental timeline. The experiment lasted 4 days in total. The green line represents the habituation days (30 minutes) for 3 consecutive days, and the red line represent the time after injection of the pharmacological agent and the blue line represent the recording time with a digital video camera after induction of itch.

Table 3.3. Experimental groups in experiment 1.

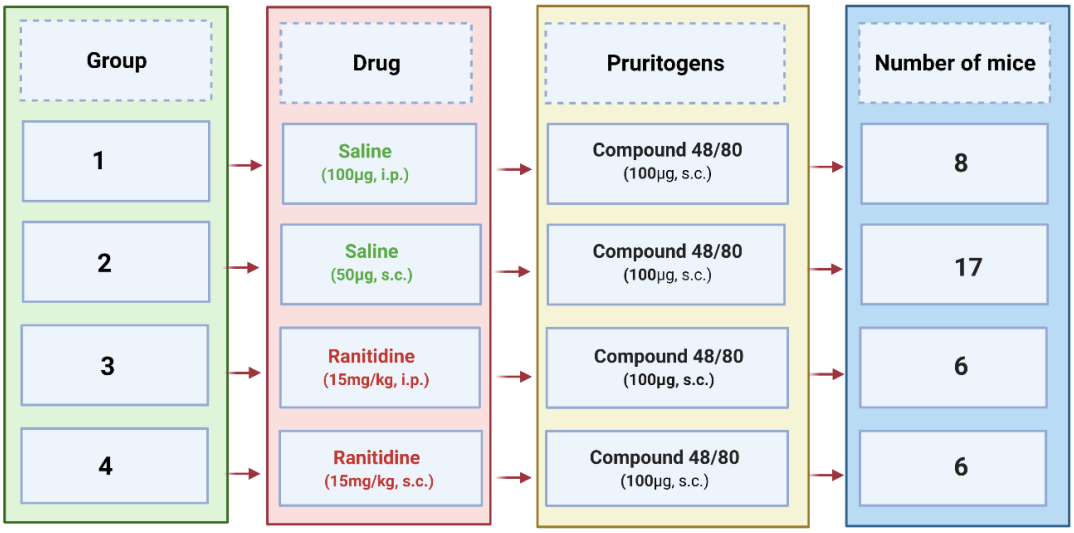


Mice were weighed and randomly assigned into 5 groups to receive either Mepyramine or vehicle; they were injected i.p. or s.c. with mepyramine at 10, 20 mg/kg body weight or vehicle (saline) solution as a control group. **(Groups 1-2)** Control group mice received i.p. or s.c. injection of vehicle in a volume of 100 µL and 50 µL respectively. **(Groups 3-4)** Mice received 10 and 20 mg/kg i.p. of mepyramine and **(Group 5)** Mice received 10 mg/kg s.c. of mepyramine. Mepyramine /vehicle was injected 30 minutes before injection of pruritogens.

Experiment 2: Role of H_2R

After 3 consecutive days of habituation mice were randomly assigned into 4 groups as indicated in Table 3.4 On the experimental day (4^{th} day), mice were weighed and habituated to a small plastic chamber for 30 minutes. Then, each mouse was administered with a different dose of ranitidine (15 mg/kg, i.p., or 15 mg/kg, s.c.), 30 minutes before pruritogens to determine systemic and peripheral local effects of H_2R antagonists on itch. Behavioural responses after ranitidine or vehicle were recorded for 40 min and analysed at 5-min intervals. Control animals received equivalent vehicle solution without ranitidine. Figure 3.3 shows the experimental timeline and Table 3.4 shows a summary of doses, sites of injection and number of animals in each experimental group.

Table 3.4 Experimental groups in experiment 2.

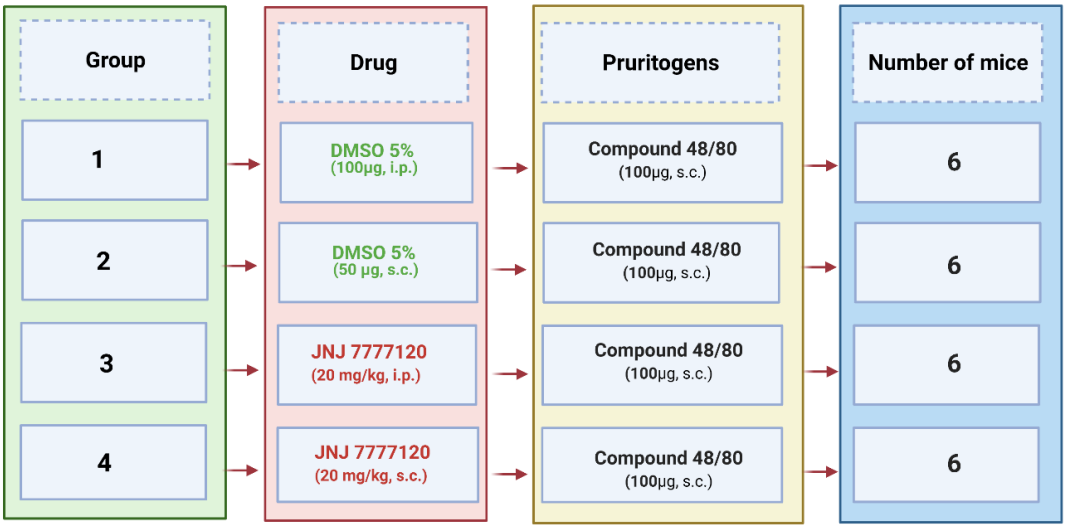


Mice were weighed and randomly assigned into 4 groups to receive either Ranitidine or vehicle; they were injected i.p. or s.c. with ranitidine at 15 mg kg/kg body weight or vehicle (saline) solution as a control group. **(Group 1-2)** Control group mice received i.p. or s.c. injection of vehicle in a volume of 100 µL or 50 µL, respectively. **(Group 3)** Mice received 15 mg/kg i.p. of ranitidine and **(Group 4)** Mice received 15 mg/kg s.c. of ranitidine. Ranitidine/vehicle was injected 30 minutes before injection of pruritogens.

Experiment 3: Role of H_4R

After 3 consecutive days of habituation mice were randomly assigned into 4 groups as indicated in Table 3.5 On the experimental day (4^{th} day), mice were weighed and habituated to a small plastic chamber for 30 min. Then, each mouse was administered with a different dose of JNJ 7777120 (20mg/kg, i.p., or 20mg/kg, s.c.) 30 minutes before pruritogen to determine systemic and peripheral effects of H_4R antagonists on itch. Behavioural responses after JNJ 7777120 or vehicle were recorded for 40 minutes and analysed at 5 minutes intervals. Control animals received equivalent vehicle solution without JNJ 7777120. Figure 3.2 shows the experimental timeline and Table 3.5 shows a summary of doses, site of injection and number of animals in each experimental group.

Table 3.5 Experimental groups in experiment 3.



Mice were weighed and randomly assigned into **4** groups to receive either JNJ777120 or vehicle; they were injected i.p. or s.c with JNJ777120 at 20mg/kg body weight or vehicle (5% DMSO) as a control group. **(Group 1-2)** Control group mice received i.p. and s.c. injection of vehicle in a volume of 100 µL and 50 µL respectively. **(Group 3)** Mice received 20 mg/kg i.p. of JNJ777120, and **(Group 4)** Mice received 20 mg/kg s.c. of JNJ777120. JNJ777120 or vehicle was injected 30 minutes before injection of pruritogens.

3.2.4.3 To determine the role of H_1R, H_2R and H_4R in histamine-mediated scratching and effects produced by Votucalis

Experiment I: Role of H_1R, H_2R and H_4R

After 3 consecutive days of habituation mice were randomly assigned into 9 groups as indicated in Table 3.6. On the experimental day (4th day), mice were weighed and habituated to a small plastic chamber for 30 min. Then, each mouse was administered with a different dose of mepyramine, (10mg/kg, s.c. , 20mg/kg, i.p.) ranitidine, (15mg/kg, s.c., 15mg/kg, i.p.) or JNJ 7777120 (20mg/kg, s.c., 20mg/kg, i.p.) were co-injected with local administration of Votucalis (10mg/kg, s.c.), 30 minutes before injection of pruritogen. Behavioural responses after combination of treatment or vehicle control were recorded for 40 minutes and analysed at 5 minutes intervals. Control animals received equivalent vehicle solution without combination drugs. Figure 3.4 shows the experimental timeline and Table 3.6 shows a summary of doses, site of injection and number of animals in each experimental group.

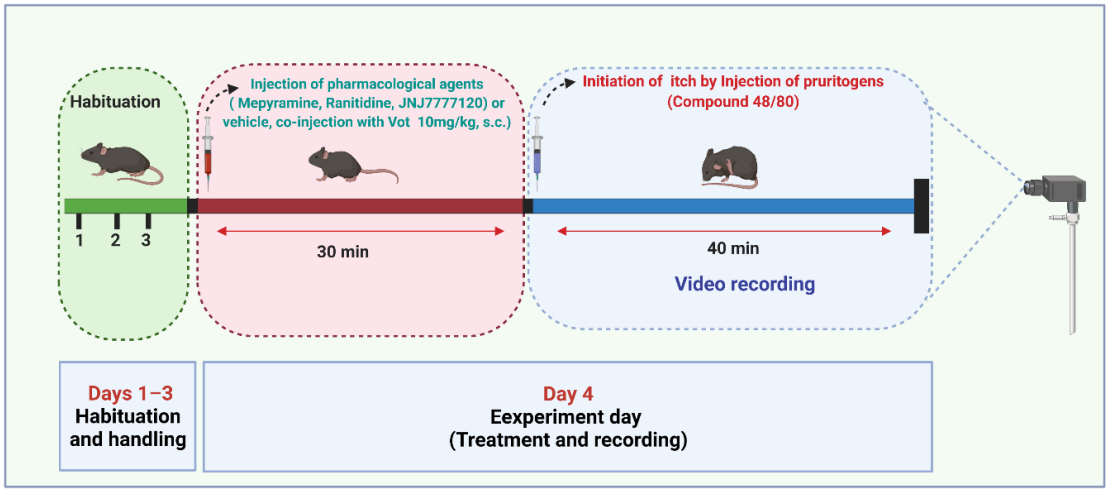
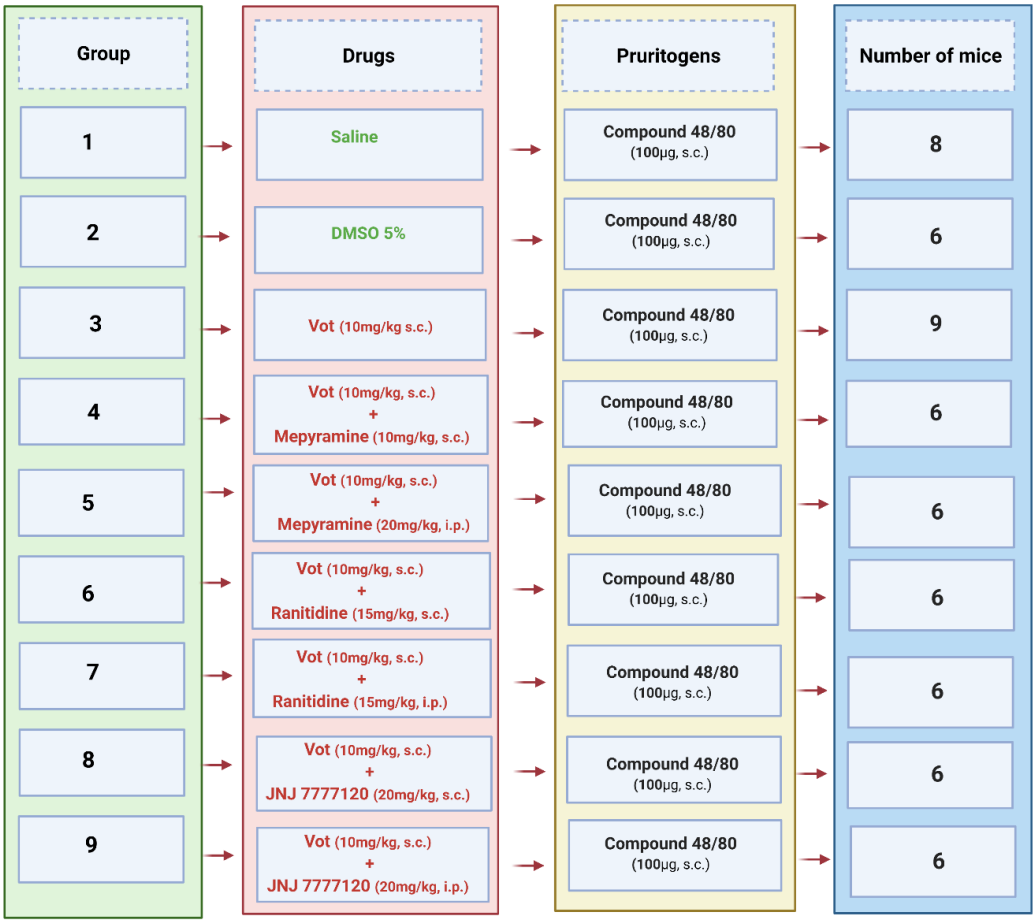


Figure 3.4. Schematic representation of the experimental timeline. The experiment lasted 4 days in total. The green line represents the habituation days (30 minutes) for 3 consecutive days, and the red line represent the time after injection of the pharmacological agent and the blue line represent the recording time with a digital video camera after induction of itch.

Table 3.6 Experimental groups in experiment 1.



Mice were weighed and randomly assigned into 9 groups to receive either a combination of Votucalis (10 mg/kg, s.c) with one of the selective H_1R, H_2R and H_4R antagonists or vehicle 30 minutes before the injection of pruritic agent. **(Groups 1-2)** Control group mice received i.p. and s.c. injection of vehicle in a volume of 100 µL and 50 µL respectively. (**Group 3**) Mice received only votucalis (10mg/kg, s.c.), **(Group 4)** Mice received combination of mepyramine (10mg/kg, s.c.) + votucalis (10mg/kg, s.c.), **(Group 5)** Mice received combination of mepyramine (20mg/kg, i.p.)+ Votucalis (10mg/kg, s.c.), **(Group 6)** Mice received combination of ranitidine (15mg/kg, s.c.)+ votucalis (10 mg/kg, s.c.), **(Group 7)** Mice received combination of ranitidine (15 mg/kg, s.c.)+ votucalis (10 mg/kg, s.c.), **(Group 8)** Mice received combination of JNJ777120 (20mg/kg, s.c.) + votucalis (10mg/kg, s.c.), and **(Group 9)** Mice received combination of JNJ777120 (20mg/kg, i.p.) + Votucalis (10 mg/kg, s.c.). Combination or vehicle was injected 30 minutes before injection of pruritogens.

3.3 Statistical analysis

All the data analysis and statistical comparisons were made using GraphPad PrismTM, (version 9.1.2) for Windows/OS (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The results of behavioural changes are presented in the graphs as mean ± SEM. Each group included 6-17 mice. Statistical analysis was performed by one- or two-way analyses of variance (ANOVA) with Bonferroni's multiple comparison post-hoc tests or by unpaired Student's t-test when two groups were compared. Repeated measures ANOVA used where drug effects were quantified repeatedly over a period of time. A value of $p < 0.05$ *vs* respective control group was considered to be statistically significant.

3.4 Results

3.4.1 Compound 48/80 and chloroquine induced scratching in mice.

As indicated in Figure 3.5, s.c. injection of pruritogens compound 48/80 (Figure 3.5 A and B) or chloroquine (Figure 3.5 C and D) induced scratching behaviour within ≈40-minute observation period when compared with vehicle control animals injected s.c. with saline. Total number of scratches for compound 48/80 was: 421.3 ± 18.9; n=17 and, chloroquine was: 461.2 ± 30.2; n=17 compared to saline treated control group (5.25± 1.4).

3.4.2 Systemic administration of Votucalis resulted in significant inhibition of histaminergic itch.

As indicated in Figure 3.6, systemic i.p. administration of Votucalis inhibited histamine-dependent itch behaviour induced by compound 48/80 over the first 20 minutes of the observation period (potentially indicating for a shorter duration of action). The effect was observed only with the two highest doses of 20 and 40 mg/kg (Figure 3.6 A and B; total number of scratches after treatment with; Votucalis 20 mg/kg: 118±12.1, 40mg/kg: 114±9.5; n=6-7 and saline: 174±12.2), while lower doses of Votucalis (1, 3 and 10mg/kg) did not show any significant differences between treated and saline control groups (P=0.01).

For the whole 40 minutes observation period, anti-itch effect produced by Votucalis was not significant when compared with the vehicle control group (Figure 3.6 A,C and D; total number of scratches after treatment with Votucalis 1 mg/kg: 266 ±38.3 ,3 mg/kg: 198.5 ±50.8, 10 mg/kg: 242 ±28.8, 20 mg/kg: 171.3±21.5, 40mg/kg: 220±42.6 and saline: 283.8±23.6; $F_{(5, 35)} = 2.36$, P=0.059; n=6-7).

3.4.3 Systemic administration of Votucalis did not inhibit histamine-independent itch.

As indicated in Figure 3.7 systemic i.p. treatment with Votucalis injected 30 minutes before pruritogens did not improve the itch behaviour induced by histamine-independent pruritogens chloroquine. This result is showing ineffectiveness of Votucalis for histamine-independent itch (Figure 3.7 A and C and D; total number of scratches after treatment with Votucalis 1 mg/kg: 450.5±96, 3 mg/kg: 452.9 ±61.4, 10 mg/kg: 421.3 ±74.2, 20 mg/kg: 405.7±54.5, 40mg/kg: 421.8±55.3, and saline: 461.5±31.3, $F_{(5, 35)} = 0.5683$, P=0.7237; n=6-9).

3.4.4 Peripheral administration of Votucalis resulted in significant inhibition of histaminergic itch.

As indicated in Figure 3.8, peripheral s.c. administration of Votucalis inhibited histamine-dependent itch behaviour induced by compound 48/80 over 40 minutes time in dose dependent manner in mice. The anti-pruritic effect of local administration of Votucalis had longer duration of action (40 minutes) in comparison with systemic administration where the anti-itch effect was only observed for 20 minutes (Figure 3.8 A and C, total number of scratches after treatment with peripheral Votucalis 0.3 mg/kg: 237.7±38.8, 1 mg/kg: 207.7±23.4, 3 mg/kg: 175.2±42.9, 10mg/kg: 168.5±14.3, 20mg/kg: 271.5±28.5 and saline: 421.3±18.9). Interestingly, the anti-itch effect produced by Votucalis was dose-dependent for the dose range between 0.3 and 10 mg/kg. However, the highest tested dose of 20 mg/kg showed decreased anti-itch effect compared to the lower doses of Votucalis tested, although the effect was still significant when compared with the saline control (Figure 3.8, D $F_{(5, 35)} = 38.55$, $P<0.0001$; n=6-9) . This observation indicates a biphasic or bell-shape dose response curve produced by peripheral administration of Votucalis.

3.4.5 Role of H_1R in histaminergic itch

As indicated in Figure 3.9, mepyramine, a first-generation selective H_1R antagonist after systemic administration at lower doses (10 mg/kg, i.p.) did not significantly reduce scratching behaviour in mice while, increasing the dose to 20mg/kg, caused a significant reduction in scratching induced by histamine-dependent pruritogenic compound 48/80 (100µg, s.c.) (Figure 3.9, $P<0.0001$). Interestingly, lower doses of mepyramine after local s.c. administration (10mg/kg) caused a significant reduction in compound 48/80-induced scratching when compared to vehicle treated mice (Figure 3.9 C and D total number of scratches after treatment with mepyramine 10 mg/kg: 149.8±24.8, and saline: 421.3±18.9, $F_{(4, 28)}= 44.75$ $P<0.0001$, n=6). The percentage of inhibition of the total number of scratches after local and systemic administration of the mepyramine were as follow: 10 mg/kg, i.p.: 9.71%, 20 mg/kg, i.p.: 70.8% and 10 mg/kg, s.c: 64.4% (Table 3.7).

3.4.6 Role of H_2R in histaminergic itch

As indicated in Figure 3.10, administration of ranitidine, selective H_2R antagonist by systemic route at (15mg/kg i.p.) did not significantly reduce histamine-dependent scratching when compared to saline-injected mice (Figure 3.10; total number of scratches after treatment with ranitidine 15 mg/kg: 367.5±28.7, saline: 470.5±32.5; P=0.1166; n=6). The same dose of ranitidine when injected peripherally s.c. significantly reduced compound 48/80-induced itch (Figure 3.10, C and D total number of scratches after treatment with ranitidine 15 mg/kg : 199±32.9, saline: 421.3±18.9; $F_{(3, 21)}$ = 19.03, P<0.0001, n=6). The percentage of inhibition of the total number of scratches by local and systemic administration of the ranitidine were as follow 15mg/kg, i.p.: 22.7% and 15mg/kg, s.c.: 52.8% (Table 3.7).

3.4.7 Role of H_4R in histaminergic itch

As indicated in Figure 3.11, peripheral s.c. and systemic i.p. administration of JNJ 7777120, selective H_4R antagonist at dose of 20mg/kg inhibited histamine-dependent itch behaviour induced by compound 48/80. This effect was observed over 40 minutes observation period in mice (Figure 3.11, C and D total number of scratches after treatment with JNJ 7777120 20mg/kg i.p. : 252.5±23.9 vs. DMSO i.p. : 414±26.4, and 20 mg/kg, s.c.: 283.33±25.79 *vs.* DMSO s.c. : 415.8±30.45; $F_{(3, 21)}$ = 25.45, P<0.001, n=6). The percentage of inhibition of the total number of scratches by local and systemic administration of JNJ 7777120 were as follow 20mg/kg i.p.: 39.01% and 20 mg/kg s.c. : 31.86% (Table 3.7).

3.4.8 Peripheral H_1R and H_2R as well as central H_4R mediated effects of Votucalis on histamine-dependent itch.

Mepyramine, when injected systemically (i.p.) required higher dose to reduce histamine-dependent itch produced by compound 48/80 as 10 mg/kg was ineffective (P>0.05; n=6), while 20 mg/kg significantly inhibited compound 48/80-induced itch (Figure 3.9; P<0.0001; n=6). In contrast, peripheral (s.c.) administration of mepyramine at the dose of 10 mg/kg was statistically effective in inhibition of compound 48/80-induced itch (Figure 3.8.; P<0.0001; n=6). Figure 3.12 indicates that peripherally (s.c.) effective dose of mepyramine produced stronger anti-itch effect when co-administered

with Votucalis on histamine-dependent itch produced by compound 48/80 (P=007), while systemic (i.p.) administration of mepyramine did not affect anti-itch effect of Votucalis (P>0.05).

Ranitidine, when injected systemically (i.p.) at the dose of 15 mg/kg did not reduce histamine-dependent itch produced by compound 48/80 (Figure 3.9; P>0.05). The same dose of ranitidine when injected peripherally (s.c.) statistically significantly reduced compound 48/80-induced itch (Figure 3.10; P<0.0001) as well as produced stronger anti-itch effect when co-administered with Votucalis on histamine-dependent itch induced by compound 48/80 (Figure 3.12; P=002), while systemic (i.p.) administration of ranitidine did not affect anti-itch effect of Votucalis (Figure 3.11).

JNJ 7777120, when injected either i.p. or s.c. at the dose of 20 mg/kg statistically significantly reduced histamine-dependent itch produced by compound 48/80 (Figure 3.11; P<0.0001 and P=0.0004 respectively). However, only systemically (i.p.) administered JNJ 7777120 produced stronger–anti-itch effect when co-administered with Votucalis on compound 48/80-induced itch (Figure 3.12; P=002), while peripheral (s.c.) administration of JNJ 7777120 did not affect anti-itch effect of Votucalis (P>0.05).

In summary, Votucalis after local administration at dose of 10mg/kg, provided inhibition of histaminergic itch around 62 %, which was further increased by both peripheral H_1R and H_2R or central H_4R antagonism up to around 85, 90 and 81 %, respectively (peripheral H_1R and H2R or central H_4R antagonism without Votucails inhibited histaminergic itch around 64, 53 and 27 %, respectively). The incomplete inhibition of itch by Votucalis may be assumed to be due to high levels of histamine released by mast cell degranulation in this model (Table 3.7).

3.4.9 Peripheral administration of Votucalis produced anti-pruritic effects at a lower dose range compared to systemic administration.

As summarized in Figure 3.13, Votucalis produced dose-dependent anti-itch effects on histamine-dependent itch produced by compound 48/80. Overall, localised peripheral (s.c.) administration of Votucalis produced anti-itch effects at a lower dose range in comparison to systemic (i.p.) administration indicating higher potency after localised peripheral Votucalis administrations observed as a left-ward shift of the dose-response curve for Votucalis.

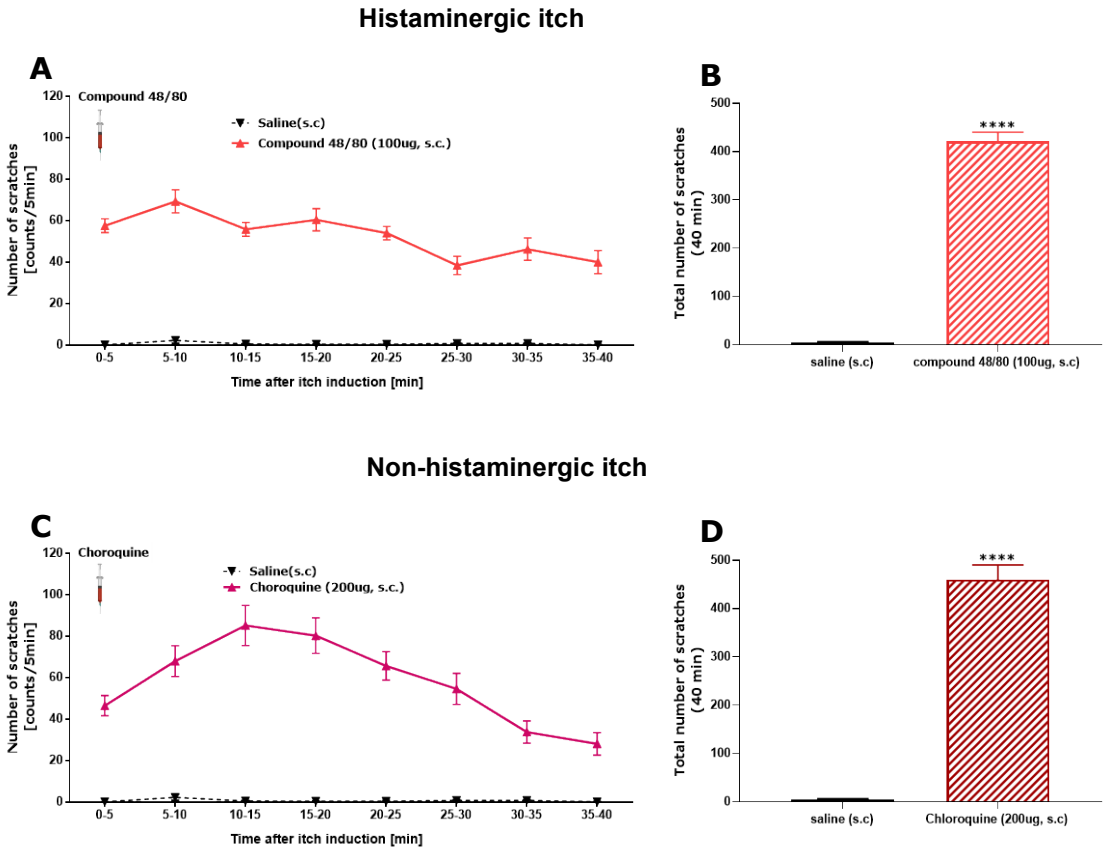


Figure 3.5 Peripheral (s.c.) administration of compound 48/80 and chloroquine evoked itch behaviour in mice. (A) Time-course effect on the total number of scratches that occurred over a 40-minute time period after s.c. injection of compound 48/80 into the nape of the mouse neck. **(C)** Time-course effect on the total number of scratches that occurred over a 40-minute time period after s.c. injection of chloroquine into the nape of the mouse neck Bar graphs displaying a total number of scratches across a 40 minutes observation period after s.c. injection of compound 48/80. **(B and D)** Bar graphs displaying a total number of scratches across a 40-minute observation period after s.c. injection of compound 48/80 (B) or chloroquine (D). Data are presented as mean ± SEM, n= 4-18 in each group. The asterisk (*) denotes significance *vs.* vehicle control animals (A and C, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B and D, un-paired Student's t-test).

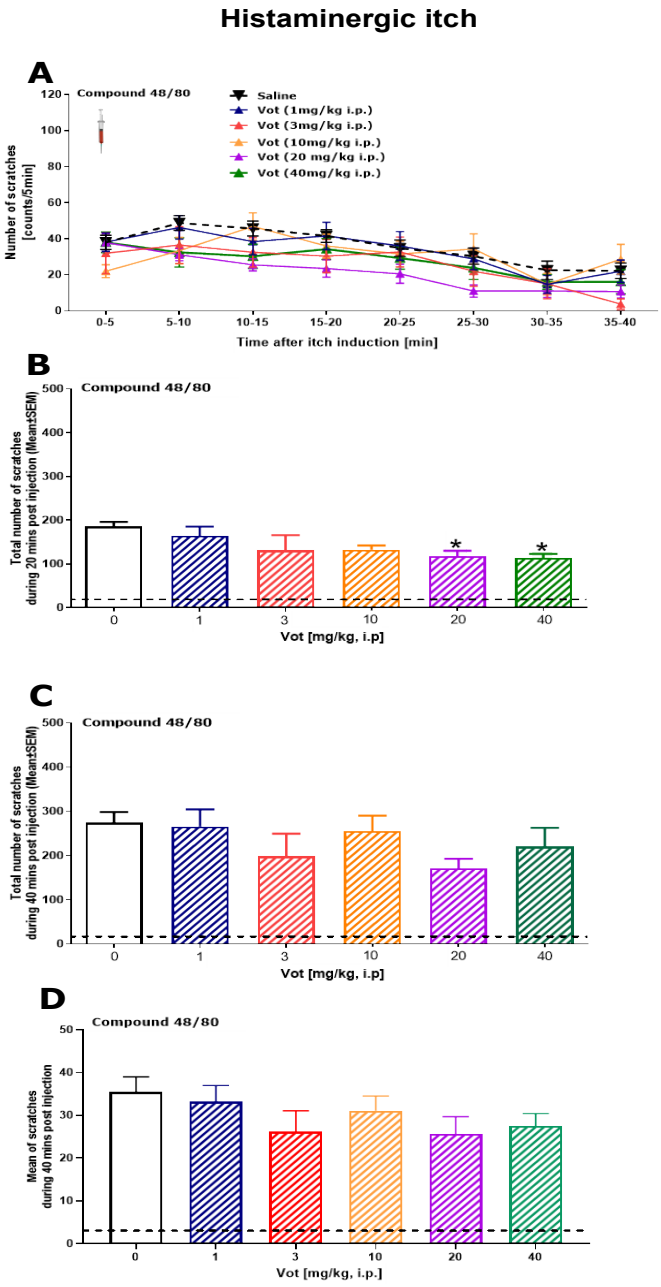


Figure 3.6 Systemic intraperitoneal (i.p.) administration of Votucalis (Vot) inhibited itch behaviour induced by histamine-dependent pruritogens over 20-minute time in mice. (A) Time-course effect of i.p. injection of Votucalis or vehicle (saline), on the total number of scratches that occurred over a 40 minutes time period after s.c. injection of compound 48/80 into the nape of the mouse neck. Mice received a systemic injection of Votucalis (Vot 1-40 mg/kg; n = 6-9) or vehicle (saline; n = 17) 30-minute before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5-min intervals for 40 min. **(B)** Bar graphs displaying a total number of scratches across the first 20-minute observation period after s.c. injection of compound 48/80 for each treatment **(C)** Bar graphs displaying a total number of scratches across a 40-minute observation period after s.c. injection of compound 48/80 for each treatment. (D) Comparison of the groups with repeated measure ANOVA followed by Bonferroni's test. Data are presented as means ± S.E.M, n = 6-17 in each group. Dotted lines in the bar graphs indicate saline induced scratching and asterisk (*) denotes significance *vs.* vehicle control animals; *p<0.05 (A, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B-C, one-way analysis of variance, followed by Bonferroni comparison post hoc test; D, repeated measure ANOVA followed by Bonferroni's test).

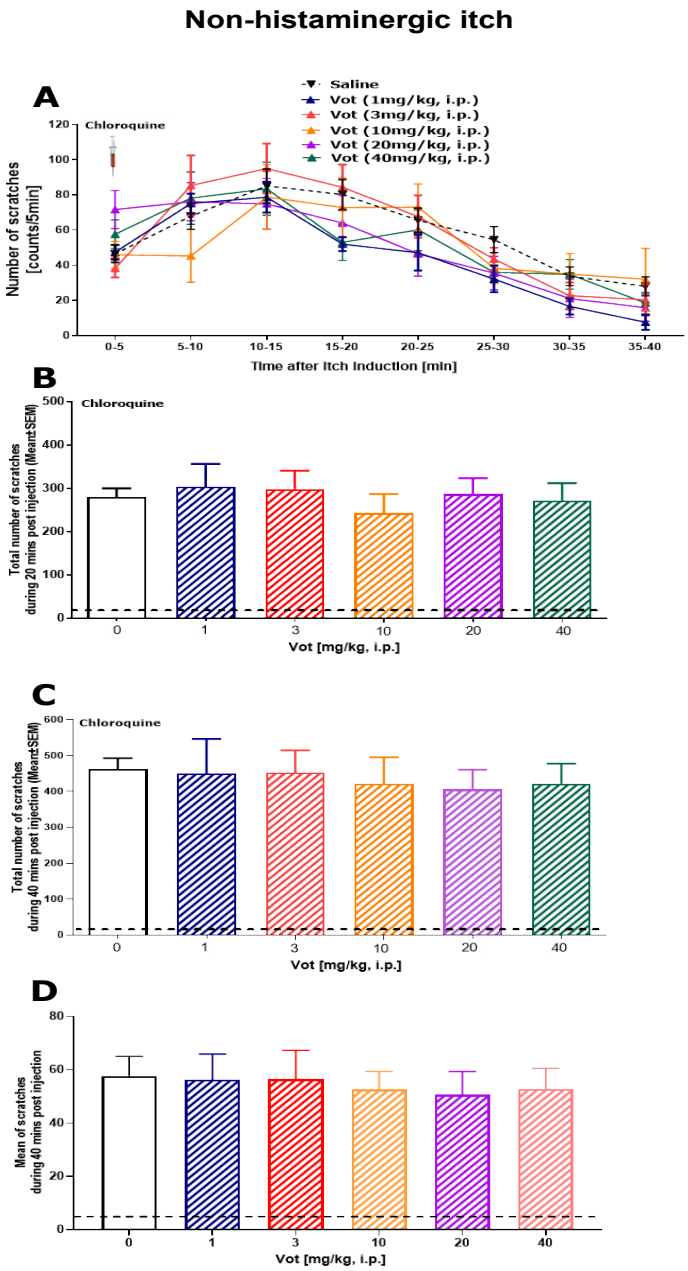


Figure 3.7 Systemic intraperitoneal (i.p.) administration of Votucalis (Vot) did not significantly reduce scratching behaviour caused by histamine-independent pruritogens (chloroquine) over 40-minute time in mice. (A) Time course effect of systemic i.p. injection of different dose of Votucalis or vehicle (saline), on the total number of scratches that occurred over a 40 minutes time period after s.c. injection of chloroquine, into the nape of the mouse neck. Mice received an i.p. injection of Votucalis (Vot , 1-40mg/kg, n = 6-7) or vehicle (saline; n = 17) 30-minute before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5-minute intervals for 40 minutes. **(B)** Bar graphs displaying a total number of scratches across the first 20-minute observation period after s.c. injection of chloroquine for each treatment. **(C)** Bar graphs displaying a total number of scratches across a 40 minutes observation period after s.c. injection of chloroquine for each treatment. **(D)** Comparison of the groups with repeated measure ANOVA followed by Bonferroni's test. Data are presented as mean ± SEM values. Dotted lines in the bar graphs indicate saline induced scratching and, the asterisk (*) represents significance vs vehicle control animals; P>0.05 (not statistically significant) (A, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B-C, one-way analysis of variance, followed by Bonferroni comparison post hoc test; D, repeated measure ANOVA followed by Bonferroni's test).

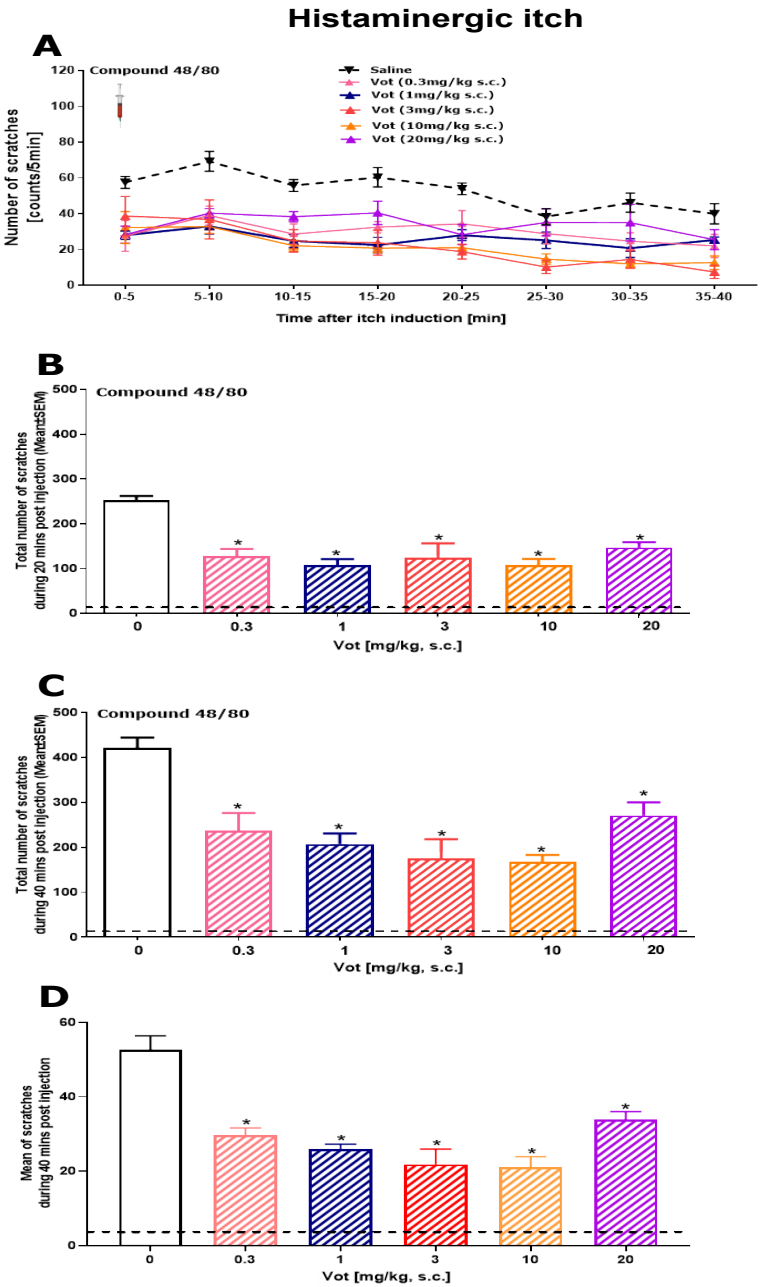


Figure 3.8 local subcutaneous (s.c.) administration of Votucalis (Vot) inhibited itch behaviour induced by histamine-dependent pruritogens over 40 minutes time in mice. (A) Time course effect of peripheral s.c. injection of different dose of Votucalis, or vehicle, on the total number of scratches that occurred over a 40 minutes time period after s.c. injection of compound 48/80, into the nape of the mouse neck. Mice received an injection of Votucalis (0.3-20 mg/kg; n = 6) or vehicle (saline; n = 17) 30 minutes before the injection of compound 48/80. Itch behaviour was recorded, and scratches were counted in 5 minutes intervals for 40 minutes. **(B)** Bar graphs displaying a total number of scratches across the first 20 minutes observation period after s.c. injection of compound 48/80 for each treatment. **(C)** Bar graphs displaying a total number of scratches across a 40 minutes observation period after s.c. injection of compound 48/80 for each treatment. **(D)** Comparison of the groups with repeated measure ANOVA followed by Bonferroni's test. Data are presented as mean ± SEM values. Dotted lines in the bar graphs indicate saline induced scratching and, the asterisk (*) represents significance *vs* vehicle control animals (A, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B-C, one-way analysis of variance, followed by Bonferroni comparison post hoc test; D repeated measure ANOVA followed by Bonferroni's test).

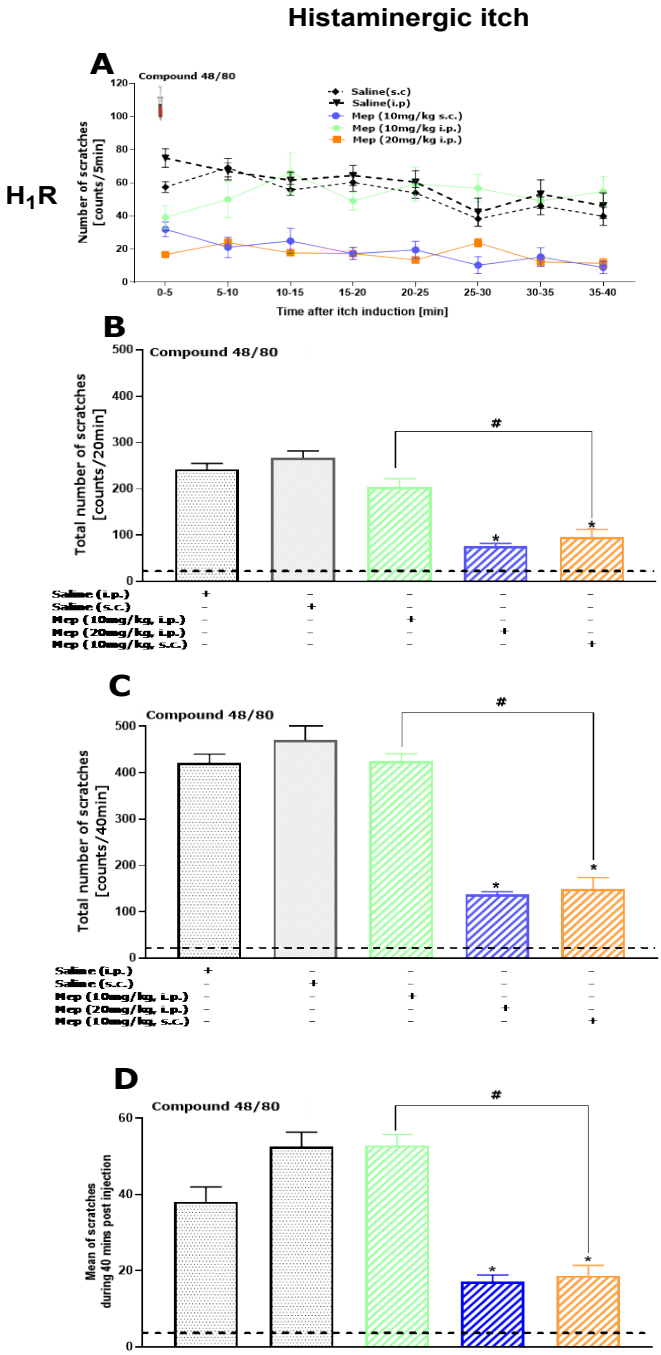


Figure 3.9 Administration of H1 receptor (H_1R) antagonist mepyramine (Mep) inhibited itch behaviour induced by histamine-dependent pruritogens in mice. (A) Time-course effect of peripheral s.c. or systemic i.p. injection of mepyramine, or vehicle (saline), on the total number of scratches that occurred over a 40 minutes time period after s.c. injection of compound 48/80 into the nape of the mouse neck. Mice received an s.c. injection of mepyramine (Mep 10-20mg/kg; i.p. or s.c.; n = 6) or vehicle (saline, n = 6-17) 30 minutes before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5-minute intervals for 40 minutes. **(B)** Bar graphs displaying a total number of scratches across the first 20-minute observation period after s.c. injection of compound 48/80 for each treatment. **(C)** Bar graphs displaying a total number of scratches across a 40 minutes observation period after s.c. injection of compound 48/80 for each treatment. **(D)** Comparison of the groups with repeated measure ANOVA followed by Bonferroni's test. Data are presented as mean ± SEM values. Dotted lines in the bar graphs indicate saline induced scratching, the number symbol (#) represents significance *vs* mice pre-treated with mepyramine s.c. and, the asterisk (*) represents significance *vs* vehicle control animals (A, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B-C, one-way analysis of variance, followed by Bonferroni comparison post hoc test; D repeated measure ANOVA followed by Bonferroni's test)

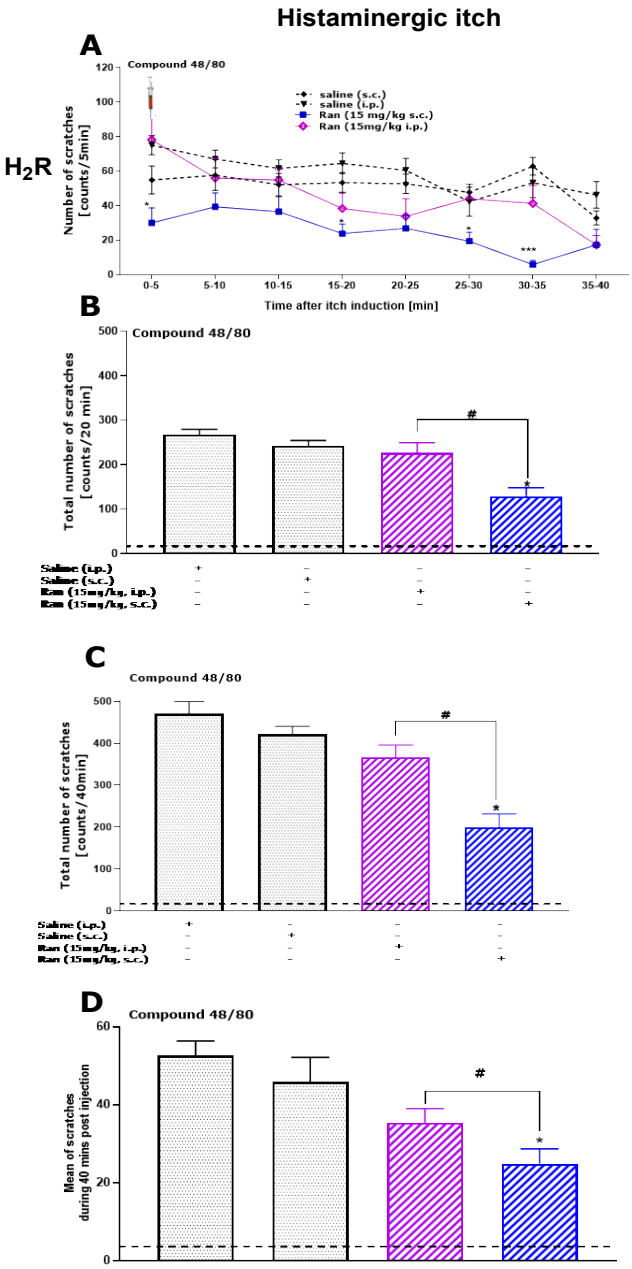


Figure 3.10 Administration of H2 receptor (H_2R) antagonist ranitidine (Ran) inhibited itch behaviour induced by histamine-dependent pruritogens in mice. (A) Time-course effect of peripheral s.c. or systemic i.p. injection of ranitidine, or vehicle (saline), on the total number of scratches that occurred over a 40 minutes time period after s.c. injection of compound 48/80 into the nape of the mouse neck. Mice received injections of ranitidine (Ran 15mg/kg; i.p. or s.c.; n = 6) or vehicle (saline, n = 6-17) 30-minute before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5 minutes intervals for 40 minutes. **(B)** Bar graphs displaying a total number of scratches across the first 20 minutes observation period after s.c. injection of compound 48/80 for each treatment. **(C)** Bar graphs displaying a total number of scratches across a 40 minutes observation period after s.c. injection of compound 48/80 for each treatment. **(D)** Comparison of the groups with repeated measure ANOVA followed by Bonferroni's test. Data are presented as mean ± SEM values. Dotted lines in the bar graphs indicate saline induced scratching, the number symbol (#) represents significance *vs* mice pre-treated with ranitidine s.c. and, the asterisk (*) represents significance *vs* vehicle control animals (A, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B-C, one-way analysis of variance, followed by Bonferroni comparison post hoc test; D, repeated measure ANOVA followed by Bonferroni's test).

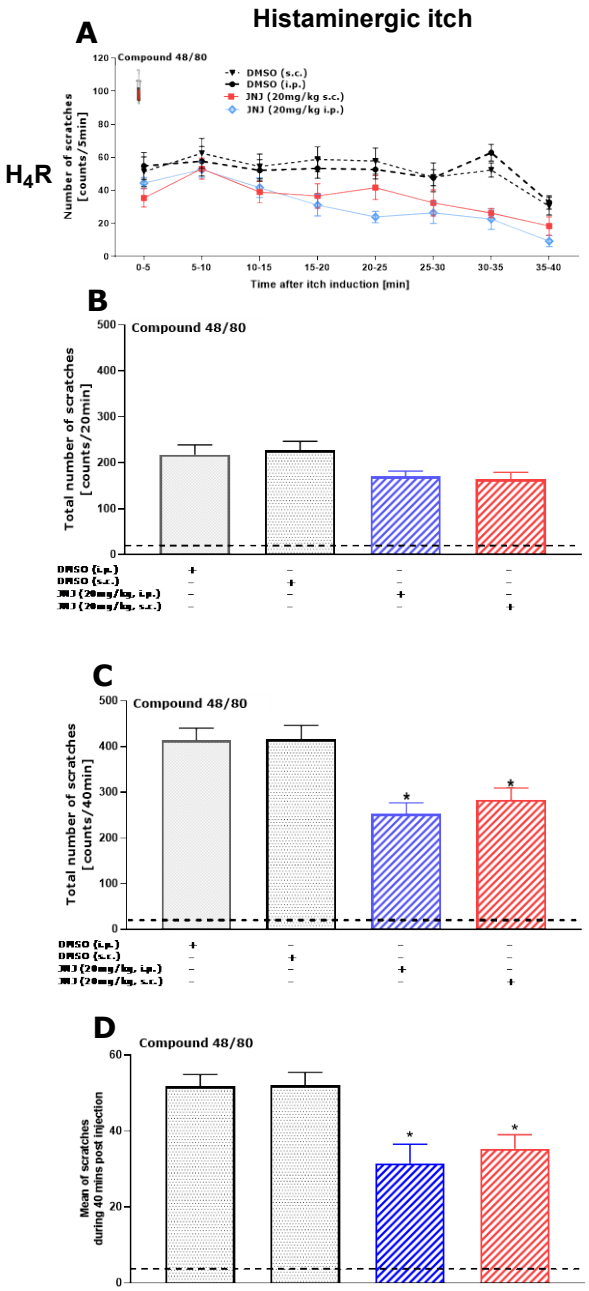


Figure 3.11 Administration of H4 receptor (H_4R) antagonist JNJ7777120 (JNJ) inhibited itch behaviour induced by histamine-dependent pruritogens in mice. (A) Time-course effect of local (s.c.) and systemic (i.p) injection of JNJ7777120, or vehicle (5% DMSO), on the total number of scratches that occurred over a 40 minutes time period after s.c. injection of compound 48/80 into the nape of the mouse neck. Mice received injection of JNJ7777120 (JNJ. 20 mg/kg; i.p. or s.c.; n = 6) or vehicle (5% DMSO, n = 6) 30 minutes before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5 minutes intervals for 40 minutes. **(B)** Bar graphs displaying a total number of scratches across the first 20 minutes observation period after s.c. injection of compound 48/80 for each treatment. **(C)** Bar graphs displaying a total number of scratches across a 40 minutes observation period after s.c. injection of compound 48/80 for each treatment. (D) Comparison of the groups with repeated measure ANOVA followed by Bonferroni's test. Data are presented as mean ± SEM values. Dotted lines in the bar graphs indicate saline induced scratching and, the asterisk (*) represents significance *vs* vehicle control animals (A, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B-C, one-way analysis of variance, followed by Bonferroni comparison post hoc test; D, repeated measure ANOVA followed by Bonferroni's test).

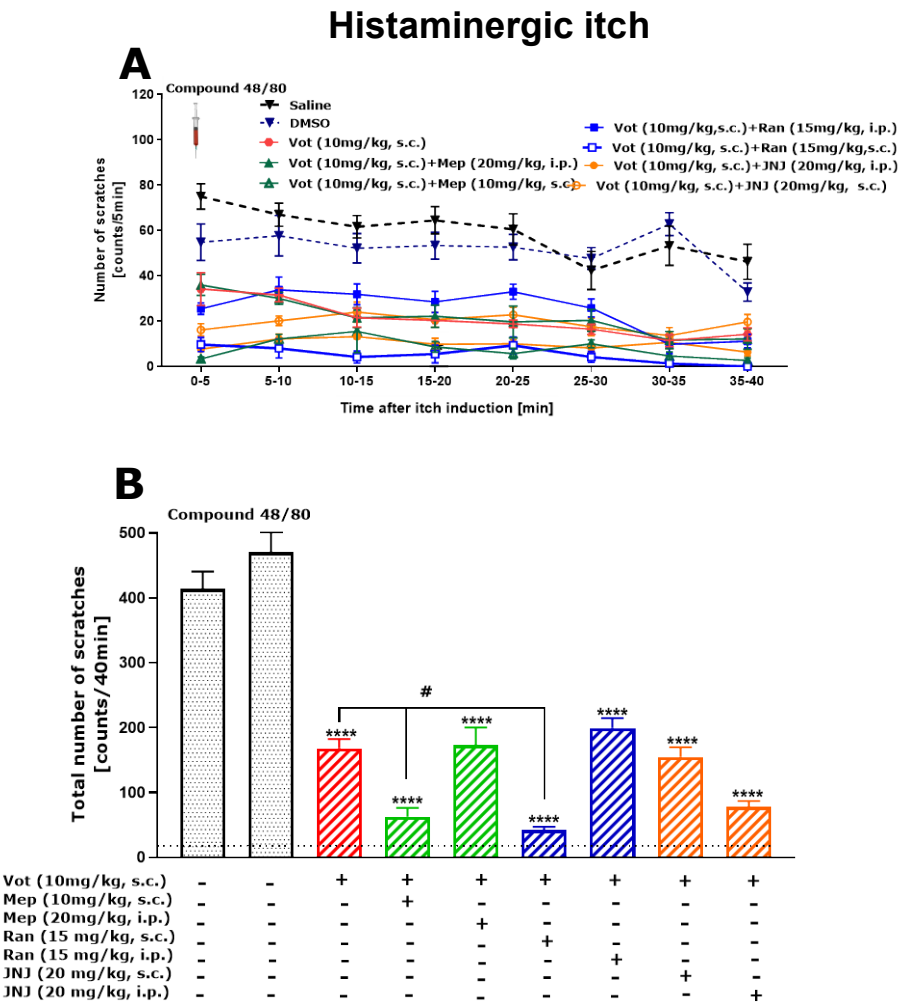


Figure 3.12 Peripheral subcutaneous (s.c.) administration of Votucalis inhibited histamine-dependent itch in mice *via* mechanism involving peripheral histamine H_1 and H_2 receptors as well as central histamine H_4 receptor. (A) Time-course effect of injection of Votucalis (Vot 10 mg/kg, s.c.), H_1R antagonist mepyramine (Mep, 10-20mg/kg, i.p. or s.c. ; n = 6), H_2R antagonist ranitidine (Ran, 15mg/kg, i.p. or s.c. ; n = 6), histamine H_4 receptor (H_4R) antagonist JNJ 7777120 (JNJ, 20mg/kg, i.p. or s.c., ; n = 6) or vehicle (saline or DMSO, n = 6-17), on the total number of scratches that occurred over a 40 minutes time period after s.c. injection of compound 48/80 into the nape of the mouse neck. Mice received a single injection of each of the drugs or a combination of Votucalis with one of the histamine receptor antagonists or vehicle 30 minutes before the injection of pruritic agent. Itch behaviour was recorded, and scratches were counted in 5-minute intervals for 40 minutes. **(B)** Bar graphs displaying a total number of scratches across 40 minutes observation period for each treatment. Data are presented as mean ± SEM values, in each group. Dotted lines in the bar graphs indicate saline induced scratching, the number symbol (#) denotes significance vs mice pre-treated with Votucalis and, the asterisk (*) denotes significance vs vehicle control animals; *P ≤ 0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001 (A, two-way analysis of variance, followed by Bonferroni comparison post hoc test; B-C, one-way analysis of variance, followed by Bonferroni comparison post hoc test).

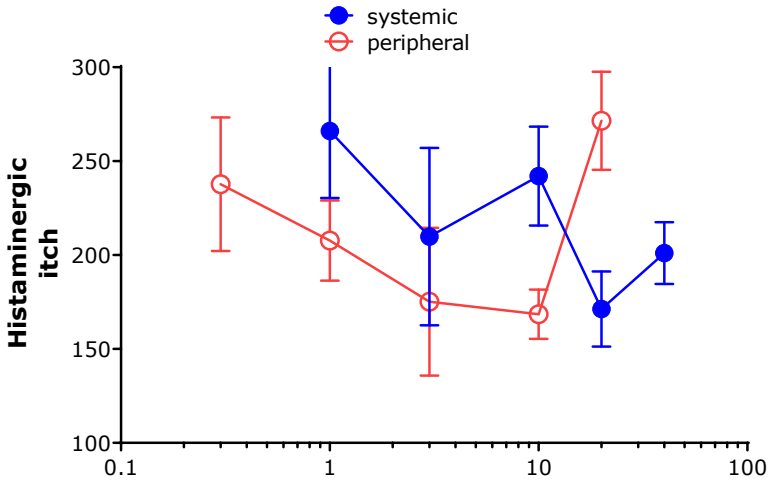


Figure 3.13 Peripheral administration of Votucalis produced anti-pruritic effects at a lower dose range compared to systemic administration. Dose-response effect of Votucalis (Vot, 0.3-40 mg/kg; intraperitoneal, i.p. or subcutaneous, s.c.) in histamine-dependent itch induced by injection of compound 48/80. Points represent the total number of scratches across a 40 minutes observation period for each treatment based on the data presented in Figure 3.5 and 3.7, which represent systemic and Peripheral effects, respectively. Data are presented as means ± S.E.M, n = 6-17 in each group.

Table 3.7. The percentage of inhibition in the total number of scratches after local and systemic administration of different histamine receptor antagonist.

Drug	Dose and route	Total number of scratches across a 40-minute observation period	percentage of inhibition across a 40-minute observation period (%)	significance *vs* vehicle control
Saline	100ul (i.p.)	470.5±32.5	0	NS
Saline	50ul (s.c.)	421.3±18.9	0	NS
DMSO 5%	100ul (i.p.)	414±26.4	0	NS
DMSO 5%	50ul (s.c.)	415.8±30.3	0	NS
Votucalis	10mg (s.c.)	168.6±13.9	62	**
Mepyramine	10 mg/kg (i.p.)	424.8±15.7	9.7	NS
Mepyramine	20 mg/kg (i.p.)	137.2±6.3	70.8	****
Mepyramine	10 mg/kg (s.c.)	149.8±24.8	64.4	****
Ranitidine	15 mg/kg (s.c.)	199±32.9	52.8	****
Ranitidine	15 mg/kg (i.p.)	363.7±26.4	22.7	NS
JNJ7777120	20mg/kg (i.p.)	252.5±23.9	39.	***
JNJ7777120	20mg/kg (s.c.)	283.3±25.8	29.7	**
Votucalis +	10mg/kg(s.c.) +20mg/kg (i.p)	173.5±27.1	63.1	****
Votucalis +	10mg/kg (s.c.) +10mg/kg (sc)	62.7±13.5	85.1	****
Votucalis + Ranitidine	10mg/kg (s.c.) +15mg/kg (i.p.)	199.5±15.0	57.6	****
Votucalis + Ranitidine	10mg/kg(s.c.) +15mg/kg (s.c.)	42.2±4.9	90	****
Votucalis + JNJ7777120	10mg/kg (s.c.) +20mg/kg (i.p.)	78.0±8.8	81.2	****
Votucalis + JNJ7777120	10mg/kg(s.c.) +20mg/kg (s.c.)	154.7±14.9	61.6	****

Not Statistically Significant (NS) p > 0.05 , Statistically significant *P ≤ 0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001

3.5 Discussion

Itch is one of the most common symptoms of skin and allergic diseases (Song et al., 2018; Weisshaar and Dalgard, 2009). In many cases, itching can result of a skin disorder, such as xerosis, psoriasis, atopic dermatitis (AD) or urticaria (Krajnik and Zylicz, 2001). In addition, itch can also arise from systemic disease such as hematologic disorder, malignancy, or renal and hepatic diseases (Krajnik and Zylicz, 2001; Tarikci et al., 2015). There is a pressing need for novel treatments for itch, especially in conditions that are not controlled by available medications in the clinical filed. Interestingly, the lack of efficacy of traditional and currently marketed antihistamines targeting H_1R in managing many itch conditions has led to the hypothesis that other HRs are involved in processing and transmission of itch (Dunford *et al*., 2007; Rossbach et al., 2011; Thurmond et al., 2017; Tatarkiewicz et al., 2019).

Targeting histamine as a strategy for the discovery of novel treatments for acute itch appears to be a very promising approach. In this study, we demonstrated that Votucalis, a novel CNS-sparing recombinant histamine binding protein, after peripheral and systemic administration produced anti-pruritic effects in mouse model of acute histaminergic itch. Both routes of administration are representing the most frequently used route of drug administration in clinical practice. Our observation, therefore, provides the first evidence for a novel strategy based on histamine capture mechanism that may potentially offer a safer and more effective strategy to control acute itch and represent a novel tool to control histamine-dependent dermatological or systemic diseases that is associated with itch.

Several studies have found that peripheral histamine plays an important role in the physiological processes connected with acute itch (Rossbach et al., 2011; Umehara et al., 2021). It has been reported that histamine is released from neuronal and non-neuronal cells such as mast cells, basophils and eosinophils at the site of nerve injury to initiate itch signaling action potentials (Khalilzadeh et al., 2018). After histamine is release, it works by bind to one of the four distinct G-protein coupled receptors (GPCRs) H_1R, H_2R, H_3R, and H_4R located on peripheral histamine-sensitive fibers mainly C-fibres (Parada et al., 2001; Farzin et al., 2002; Cannon et al., 2007). This interaction between

histamine and receptors leads to the development of itch and other histamine-mediated symptoms (Dunford *et al.*, 2007; Rossbach et al., 2011). Therefore, targeting histamine itself by using Votucalis or histamine receptors using selective receptors antagonist.

Histamine capture method after local peripheral administration of Votucalis may be superior to the antagonism of single type of receptors because in most of itch cases more than one receptor may be involved and Votucalis by preventing histamine binding to the receptors reduces the problem of targeting one or more receptors. Data showed here may suggest that, using a compound such as Votucalis which prevents the activation of all four histamine receptors may be better as anti-pruritic effect than HRs antagonist driven approach.

3.5.1 Votucalis attenuated histaminergic itch

The first part of this study was focused on identification of the anti-pruritic effect of Votucalis after localized peripheral and systemic application. The main finding is that both routes of administrations of Votucalis attenuated itching that was associated with activation of a histamine-dependent mechanism. Histamine is the most well-known endogenous and exogenous itch inducer. Many human and rodent studies have shown that intradermal injection of histamine produced itch (Heyer et al., 1997; Laidlaw et al., 2002). Also, compound 48/80, used in this current study, is a well-known inducer of histamine-dependent itch (Inagaki et al., 2002; Obara et al., 2015). Indeed, intracutaneous injection of compound 48/80 has been reported to cause mast cells degranulation resulting in histamine release from human, dog, and rodent cutaneous mast cell in a concentration-dependent fashion *via* a mechanism involving activation of PLC, increase in intracellular Ca^{2+}, and activation of PKC (Barrett et al., 1985; Tomoe et al., 1992). Therefore, the scratching behaviour induced by compound 48/80 is due to the release of histamine from the skin mast cells, and since the structure of Votucalis showed a high affinity site for histamine binding (Paesen et al., 1999; Paesen et al., 2000), it is highly likely that Votucalis neutralised endogenously released histamine resulting in a potent and dose-dependent anti-itch effect. The anti-itch effect of this compound was particularly profound after localized peripheral administration.

Specifically, delivering Votucalis directly to the area affected by itch was effective for an observation period of 40 minutes and the anti-itch effect was recorded at a 60-fold lower dose compared to the lowest systemic dose. Moreover, the systemic dose was effective only during the first 20 minutes from induction of itch. The striking difference in the potency and efficacy of Votucalis observed when the drug was administered directly to the area of itch sensation *vs.* systemically, further emphasizes the nature of Votucalis, as a non-brain-penetrating, highly potent histamine scavenger.

It seems likely that the ability of Votucalis to sequester histamine, subsequently suppressed the binding of histamine to all histamine receptors potentially leading to decrease in firing and excitability of the itch-specific primary afferents, in particular Aδ- and C-fibres, that resulted in attenuation of histaminergic itch (LaMotte et al., 2014; Obara et al., 2015). To further confirm that the anti-itch effect of Votucalis was mediated by neutralisation of histamine, we identified that Votucalis did not reduce scratching behaviour caused by chloroquine (a MrgprA3 agonist) showing its ineffectiveness for histamine-independent itch.

However, it should be noted that the highest dose of Votucalis used to inhibit itch was less effective compared to the efficacy of lower doses of Votucalis, which potentially revealing a biphasic or bell-shape dose response curve produced by Votucalis. Given that histamine plays very diverse physiological roles, this observation may suggest that higher doses of Votucalis may activate mechanisms that conversely promote itch sensation and therefore counteract the scavenging ability of Votucalis. Other explanation for that effect may be due to potential involvement of H_3R as inhibition of this receptor may result in itch effect. These results may require further investigation to study the exact cause of this bell-shape effect.

3.5.2 Peripheral H_1R and H_2R as well as central H_4R mediated itch.

The second part of experiments in this chapter was focus on the investigation of the potential role of the HRs in the transmission of itch, using the potent and widely used by researchers HR antagonists.

H_1R antagonists are the first-line pharmacologic agent for the symptomatic treatment of acute itch (Kam and Tan, 1996). However, their therapeutic efficacy is limited as many clinically significant forms of itch such as AD are not blocked by H1R antagonists (Shim and Oh, 2008; Davidson and Giesler, 2010), indicating that different histamine receptors may be involved in the mediation of itch (Kollmeier et al., 2014). Here we used selective HRs antagonists and choose different routes of their administration to distinguish the role of peripheral and central HRs in acute itch. Our study showed that histaminergic itch is predominantly mediated by peripheral H_1R, and to a lesser extent mediated by central H_1R, as peripherally administered selective antagonists of this receptors inhibited histaminergic itch at doses that were not effective with systemic administration. These conclusions may provide new scope about the mechanism of the peripheral H_1R mediated anti-itch effect as some studies have argued that the anti-itch effect resulting from H_1R antagonism is due to sedation, rather than the direct blockade of H_1R on sensory neurons (Bell et al., 2004; Imaizumi et al., 2003). In line with our observation, both mRNA and functional expression of H_1R was shown on peripheral neurons displaying characteristics of C-fibers (Kashiba et al., 2001; Rossbach et al., 2011). In addition, Bell et al. (2004) found that intradermal administration of a H_1R agonist caused dose-dependent scratching in mice.

Interestingly, however, H_1R antagonists incompletely abolished scratching behavior as we observed about 64.4% inhibition of itch. Several studies have also reported findings similar to ours showing that blocking of H_1R either failed or incompletely stopped scratching in some types of itch (Dunford et al., 2007; Rossbach et al., 2011). These results suggest that different histamine receptors could be involved in the stimulation of scratching behaviour. Remarkably, mice pre-treated with the combination of mepyramine (H_1R antagonists) and Votucalis showed less scratching during the treatment phase comparing to each drug alone, and that suggested role of

peripheral H_1R in itch transmission, was further highlighted by the use of Votucalis as the anti-itch effects was stronger when antagonists targeting peripheral H_1R were co-administered with the drug.

The role of the H_2R in itch is still somewhat controversial. In previous preclinical studies, it was shown that stimulation of H_2R by using H_2R agonist, dimaprit, failed to cause scratching behaviour (Shim and Oh, 2008). Moreover, systemic administration of the selective H_2R antagonist did not affect histamine-induced scratching (Bell et al., 2004). Similar to this outcome we also found that systemic administration of ranitidine did not affect histamine-induced itch, while targeting peripheral H_2R by using s.c. route of ranitidine caused a significant reduction in histamine dependent scratching when compared to vehicle treated mice. This outcome suggests that peripheral H_2R plays an important role in histaminergic itch.

In line with our observation, mRNA expression of H_2R was shown on primary afferent and DRG neurons in rodents (Kajihara et al., 2010; Yue et al., 2014) while their functional expression has not yet been confirmed (Rossbach et al., 2011). Interestingly, however, same to the H_1R antagonists we found that local application of ranitidine incompletely abolished scratching behavior in mice as we observed about 52.8% inhibition of itch. These findings suggest that various histamine receptors may contribute to the stimulation of scratching behavior.

Interestingly, we found that local administration of the selective H_2R antagonist produced stronger anti-itch effect when co-administered with Votucalis by 28% hence, Votucalis alone provided inhibition of histaminergic itch around 62%, which was further increased by coadministration with peripheral H_2R antagonism up to around 90%. Accordingly, we can assume that peripheral H_2R receptors might be largely involved in the regulation of histaminergic itch.

The involvement of H_4R in itch perception is becoming more evident (Paus et al., 2006; Bell et al., 2004). In 2007, Dunford et al. found that selective H_4R agonist induced itch in a dose-dependent manner, similar to histamine (Dunford et al., 2007). Interestingly, H_4R agonist induced pruritus was completely blocked by oral and i.p. dosing of the H_4R

antagonist (Dunford et al., 2007; Kamo et al., 2014). A number of researchers have reported that local administration of H_4R agonists can trigger scratching, which was inhibited by H_4R antagonists were found to be unaffected in mast cell-deficient mice (Thurmond et al., 2014; Dunford et al., 2007; Cowart et al., 2008). In addition, some researchers noticed that histamine-induced itch is reduced in H_4R-knockout mice (Xie and Li, 2019). In line with other findings, we also found that antagonism at H_4R reduced histaminergic itch. This effect was seen after both peripheral and systemic administration of a H_4R antagonist JNJ 7777120 on histamine-induced itch. Interestingly, however, H_4R antagonist when administered systemically, but not locally at peripheral site, produced stronger anti-itch effect when co-administered with Votucalis. We therefore propose that central H_4R may be largely involved in the regulation of histaminergic itch.

Taken together, the findings observed in this chapter provide the first evidence for targeting the peripheral histamine system by a recombinant histamine binding protein as a novel strategy for the control of acute itch. In addition, our findings provide further new evidence for an important involvement of peripheral H_1R and H_2R receptors and central H_4R in the regulation of histaminergic itch. Given the peripheral activity and efficacy of Votucalis, our current observations may suggest that Votucalis prevents binding of histamine to peripheral H_1R and H_2R that result in anti-pruritic efficacy of Votucalis. However, additional blockade of central H_4R may lead to the potentiation of peripheral anti-pruritic activity of Votucalis. The result of this chapter opens the door for another question about the importance of the histamine system in the modulation of different peripheral disorders such as peripheral neuropathies.

Effect of local peripheral administration of a photo- switchable H_4R agonist VUF16129 on histaminergic itch

4.1 Introduction

In the previous chapter, the role of histamine and HRs in the acute itch has been demonstrated based on the animal studies using a model of histaminergic itch and the histamine binding protein Votucalis which is design to target histamine itself *via* scavenging of its endogenous content. We also, extended our observations and determined the role of H_1R, H_2R and H_4R in acute itch by using conventional HRs antagonists. Here, for the first time we are targeting the peripheral histamine system by using a photo-switchable ligands designed to bind to H_4Rs in an animal model of itch to identify a novel strategy to study the role of H_4Rs in the mediation of histaminergic itch. This novel approach might provide opportunities to better understand various roles of H_4R in itch transmission as well as processing of sensory sensation in general.

As mentioned previously, histamine and its receptors play an important role in the transmission of itch signal. Targeting these receptors using selective ligands helps to control acute itch (Bell et al., 2004; Rossbach et al., 2011). H_4R is the most recently identified member of the HRs family and it was cloned in 2000-2001 (Oda et al. 2000). The receptor was given the fourth member among the HRs group and, besides histamine H_1R, H_2R and H_3R it also regulates the physiological and pathological functions of histamine (Thurmond et al., 2017). H_4R is a GPCRs, which is associated with Gi/o proteins and consequently down-regulates cAMP signaling, enhance the calcium mobilization and stimulates phosphorylation of MAP kinases (Cogé *et al.*, 2001; Hofstra *et al.*, 2003; Zhang *et al.*, 2007).This type of receptor share approximately 35% amino acid identity with H_3R, but it shows limited homology (much lower), around 19% with another two type of HRs such as H_1R and H_2R (Cogé *et al.*, 2001; Zhu *et al.*, 2001; Ahmad *et al.*, 2014). H_4Rs were initially identified in immune cells including eosinophils, basophil, mast cells, monocyte, dendritic and T cells (Liu et al., 2001; Buckland et al., 2003; Schirmer and Neumann, 2021). In these cells, H_4R modulates a wide range of immunity and

inflammatory responses by influencing production, stimulation and migration of cytokine and chemokine and other pro-inflammatory mediators (de Esch et al., 2005; Ahmad et al., 2014). Based on of distribution on immune cells, in addition to its proven role in immunity and inflammatory functions, the H_4R seems to be a therapeutic target for the treatment of a wide range of inflammatory and immune disorders such as asthma (Salcedo et al., 2013), allergy (de Esch et al., 2005), pain (Wolińska et al., 2017), allergic rhinitis (Takahashi et al., 2009), irritable bowel disease (Fabisiak et al., 2017), cancer(Nicoud et al., 2019) and itch (Dunford et al., 2007). H_4R is also expressed on C-afferent fiber terminals and on the DRG neuron (Rossbach et al., 2011; Thurmond et al., 2015; Wang et al., 2021), that are associated with vital importance of H_4R in transmission of itch signals (Rossbach et al., 2009; Rossbach et al., 2011).

Expression of H_4R is increased under effect of wide range of inflammatory mediators such as Interferons (IFN), tumor necrosis factor- alpha (TNF-α) and interleukin (IL-6, IL-10, IL-13), which results in the inhibition of cAMP and stimulation of mitogen-activated protein kinases (MAPK) signaling pathway (Branco et al., 2018). Mast cells and eosinophils respond to the activation of H_4R by chemotaxis, resulting in an accumulation of inflammatory cells and control of cytokine secretion by dendritic cells (DCs) and T lymphocytes. Moreover, H_4R is also involved in the increased secretion of IL-31 by T helper type 2 (Th2) cells, which plays a critical role in the development of itch sensation (Gutzmer et al., 2009; Branco et al., 2018).

The involvement of H_4R in histamine-induced itch is turning more evident (Paus et al., 2006; Bell et al., 2004), since stimulation of the peripheral H_4R using selective agonist caused scratching behavioural in mice in dose-dependent manner (Bell et al., 2004), and pre-treatment with H_4R antagonists decrease the itch response induced by both selective H_4R agonists and histamine (Dunford et al., 2007; Marson, 2011). Pre-clinical studies have shown administration of JNJ7777120 (H_4R antagonist) in mice reduced itch caused by intradermal administration of histamine, compound 48/80, and Immunoglobulin E (IgE) with the inhibitory effect being more than that shown for H_1R antagonists (Dunford et al., 2007). Despite of the presented studies, the cellular signalling mechanisms involved in itch are complicated and poorly understood and the role of H_4R in itch remains less examined in both pre-clinical and clinical trials.

H_4Rs remain exciting an opportunity for future inquiries especially since H_4R antagonists are now under clinical development and trials in different pathological conditions such as rheumatoid arthritis, asthma, psoriasis and atopic dermatitis (AD) (Thurmond et al., 2017; Werfel et al., 2019; Wang and Kim, 2020).

Selective and potent ligand agonists are required as pharmacological tools to study the involvement of receptors in physiological and *pathological conditions* in both pre-clinical and clinical studies. However, many H_4R agonists, which were identified and used in the pre-clinical models were found to exhibit *only weak* or insufficient H_4R selectivity (Igel et al., 2010; Acton, 2013). Furthermore, evaluation of affinity, potency and selectivity of several H_4R agonists in animal models is affected by species-dependent differences, especially when compared with human and rodent receptors (Igel et al., 2010; Acton, 2013). Based on this, it is clear that there is a need to understand the detailed function of a new selective H_4R ligand. Photo-pharmacology is gaining traction as a promising method for improving the spatiotemporal regulation of drug activities for their intended targets. This approach allows to use particular ligands and modulate their activity under the effect of a specified wavelength of light in order to optically control the activity of the specific type of receptor (Lerch *et al.*, 2016; Zeng *et al.*, 2021). Under light the photo-switching ligand may absorb a photon, causing a photo-chemical process that induces structural rearrangements which can alter the biological activity of the ligands that contact with their receptor (Ricart-Ortega *et al.*, 2020).

The use of light as an external trigger is utilized to alter ligand shape and structure which lead to change in the pharmacological characteristics of the ligand (Szymanski et al., 2013; Hauwert et al., 2019). This approach offers a number of advantages over the several classical drugs, such as reduction of off-target activity and systemic side effects as well as it reduces the chance to develop resistance to the drugs during treatment (Lerch et al., 2016; Gazerani, 2017). In addition, photo-pharmacology aims to develop a pharmacological agent with high site specificity and increased drug localization in target organ or tissue (Velema *et al.*, 2014; Zeng *et al.*, 2021).

Over the past decade, photo-pharmacology has been providing a new strategy by using photo-switchable ligand molecules to study *in vivo* and *in vitro* biological processes (Hüll et al., 2018),

including chloride ion channels (Stein et al., 2012), enzyme-linked membrane receptors (Hüll et al., 2018) and GPCRs (Westphal et al., 2017; Hauwert et al., 2019). Recently, several GPCRs have been effectively targeted by using photo-pharmacology methods, for example μ-opioid receptor (MOR) (Schönberger and Trauner, 2014), cannabinoid receptor 1 (CB1) (Westphal et al., 2017), metabotropic glutamate receptors (mGluR) (Levitz *et al.*, 2017), chemokine receptor (CXCR3) (Gómez-Santacana et al., 2018) β2-adrenoceptors (β2-AR) (Duran-Corbera et al., 2020) and H_3R (Hauwert et al., 2019).

The current work is aimed to study the involvement of H_4R in acute itch by using for the first time H_4R photo-switchable agonist. This unique agonist, *trans*-VUF16129 (Figure 4.1), can photo-switching to other isomer (*cis*) differing in their affinity, potency, and efficacy to H_4R upon the application of light (data not shown).

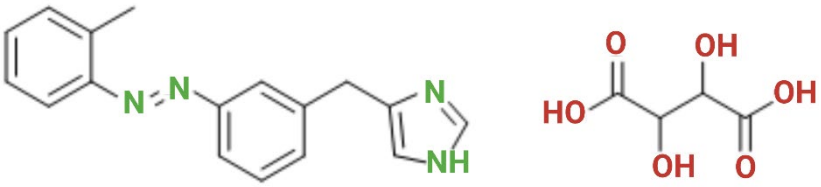


Figure 4.1 Molecular structure of *trans*-VUF16129.

The *trans* form of VUF16129 shows properties of selective H_4R agonist and therefore when injected i.d. into the nape of the mouse neck it caused scratching of the injected area; the behaviour is defined as itch behaviour associated with activation of the histamine system. This study, using this model, allowed us to validate the potential utility of locally administrated H_4R ligands to further understand the involvement of H_4R in acute itch. The knowledge gained can provide evidence for the novel therapeutic strategy that can be further tested in the clinical setting.

4.2 Materials and Methods:

4.2.1 Subjects

Experiments in this chapter were carried out using adult male C57BL/6J mice (8 weeks of age; 20–25 g; Charles River Laboratories, Harlow, UK) as previously described in Chapter two (Section 2.2). The itch-inducing procedure, behavioural assessment and measurement of scratching in this Chapter were as previously described in chapter two

(Section 2.4.2), unless otherwise stated. A schematic diagram of this experiment is shown in Figure 4.3.

4.2.2 Preparation and administration of drugs

trans-VUF16129, *cis*- VUF16129 and bavisant (also known as BEN 2001 or JNJ 31001074) were a kind gift from Professor Rob Leurs (Vrije University, Amsterdam, Netherlands). The *trans*-VUF16129 is photo-switchable compound supplied as solid orange powder stable at room temperature and stored under dark conditions. After dissolving, the compound is able to react with light leading to isomerization. To prevent the photo-isomerization we perform the handling of the compound in a dark room (under red light). Selective H_4R antagonist JNJ 7777120 was obtained from Tocris Bioscience (UK).

Local peripheral intradermal (i.d.) administration. Mice were weighed and randomized to receive either *trans*-VUF16129 or vehicle (saline). They were injected i.d. with *trans*-VUF16129 at 0.003, 0.03, 0.3, 3 and 10 µmol or vehicle (saline) solution without *trans*-VUF16129 as a control group.

To determine the role of H_4R in histamine-dependent itch induced by *trans*-VUF16129 in mice. Selective H_3R antagonist, bavisant at 10 mg/kg (0.59 µmol), i.p. and a selective H_4R antagonist JNJ 7777120 at 20 mg/kg (1.8 µmol), i.p. were administrated 15 minutes before injection of *trans*-VUF16129. All chemicals were dissolved immediately before injections (i.d., s.c.) in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK), with the exception of JNJ 7777120 which was dissolved in DMSO (dimethyl sulfoxide, 5%; Sigma-Aldrich, UK). Control animals received equivalent vehicle (saline or 5% DMSO) injections. All drugs were injected in a volume of 100 µl (i.p.) and volume 50 µl (i.d. or s.c.).

4.2.3 UV 434 and 365 nm LEDs lamp

UV-A at 365 nm was used to induce the photo-isomerization of the ligands from *trans* to *cis*. Exposure to UV-A light (365 nm, no emission in UV-B or UV-C region), was delivered by the UV-A emitting equipment installed on the top of testing box where the mouse was placed (build and tested by the group of Professor Rob Leurs at Vrije University, Amsterdam, Netherlands). UV-A exposure lasted up to 20 minutes and will not exceed 130 Jm^2 within an 8 hours period, this dose is considered safe for humans

(maximum accepted dose is 10,000 Jm2 within an 8 h period). UV-LEDs at 430 nm (equipment build and tested by the group of Professor Rob Leurs at Vrije University, Amsterdam, Netherlands) was used to reverse the effects of 365 nm light on the ligand and therefore allowed to turn *cis*- VUF16129 to *trans*- VUF16129. Figure 4.2 demonstrates experimental set up used in this experiment.

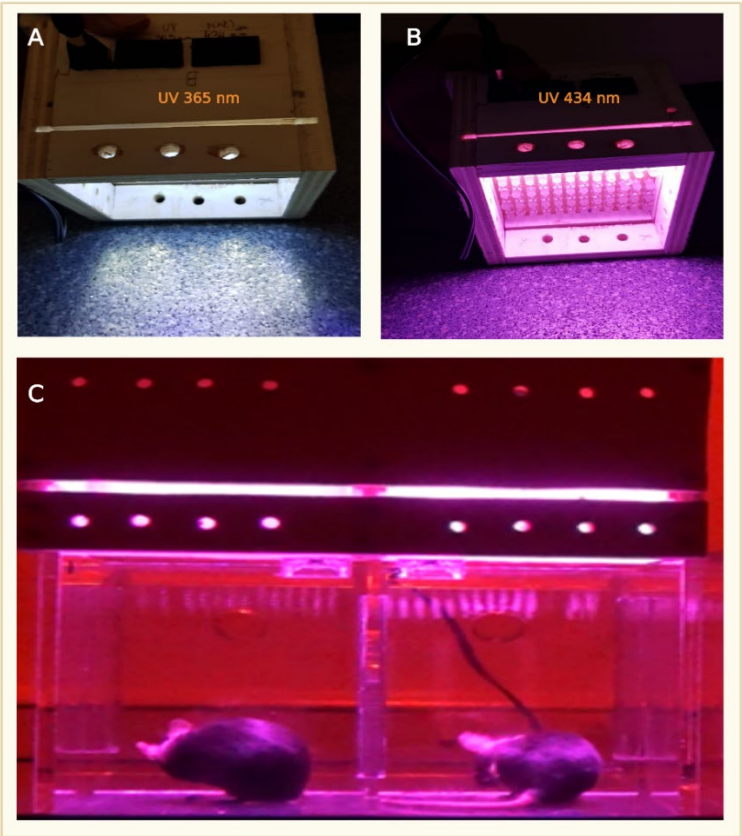


Figure 4.2 Experimental set up used in the photo-switchable experiment. (A) UV-A (365 nm) was used to induce the photo-isomerization of the ligands from *trans- VUF16129* to *cis*- VUF16129. **(B)** UV-LEDs (434 nm) was used to reverse the effects of 365 nm light on the ligand and therefore allowed to turn *cis*- VUF16129 to *trans*- VUF16129. **(C)** Represents the placement of the UV lamp on the top of testing box where the mouse was placed.

4.2.4 Itch model

4.2.4.1 Induction of itch and behavioural assessment

The method for the induction, recording and assessment of scratching behaviour is described in detail in Chapter 2.

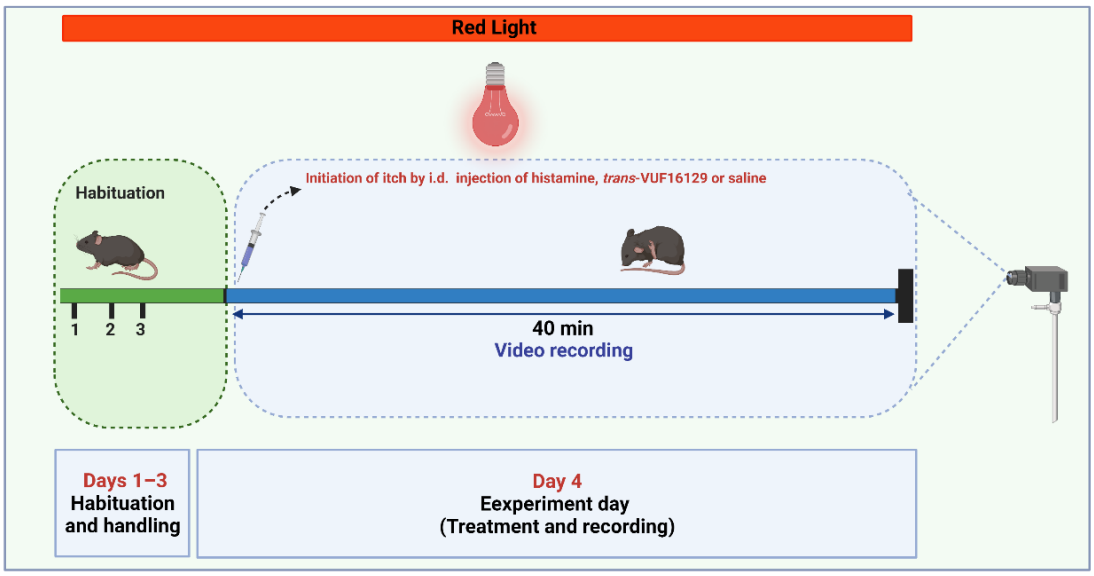


Figure 4.3 Schematic representation of the experimental timeline. The experiment lasted 4 days in total. The green line represents the habituation days (30 minutes) for 3 consecutive days and blue line represents the recording time with a digital video camera after induction of itch. The above red line indicates that the experiment was conducted under red light.

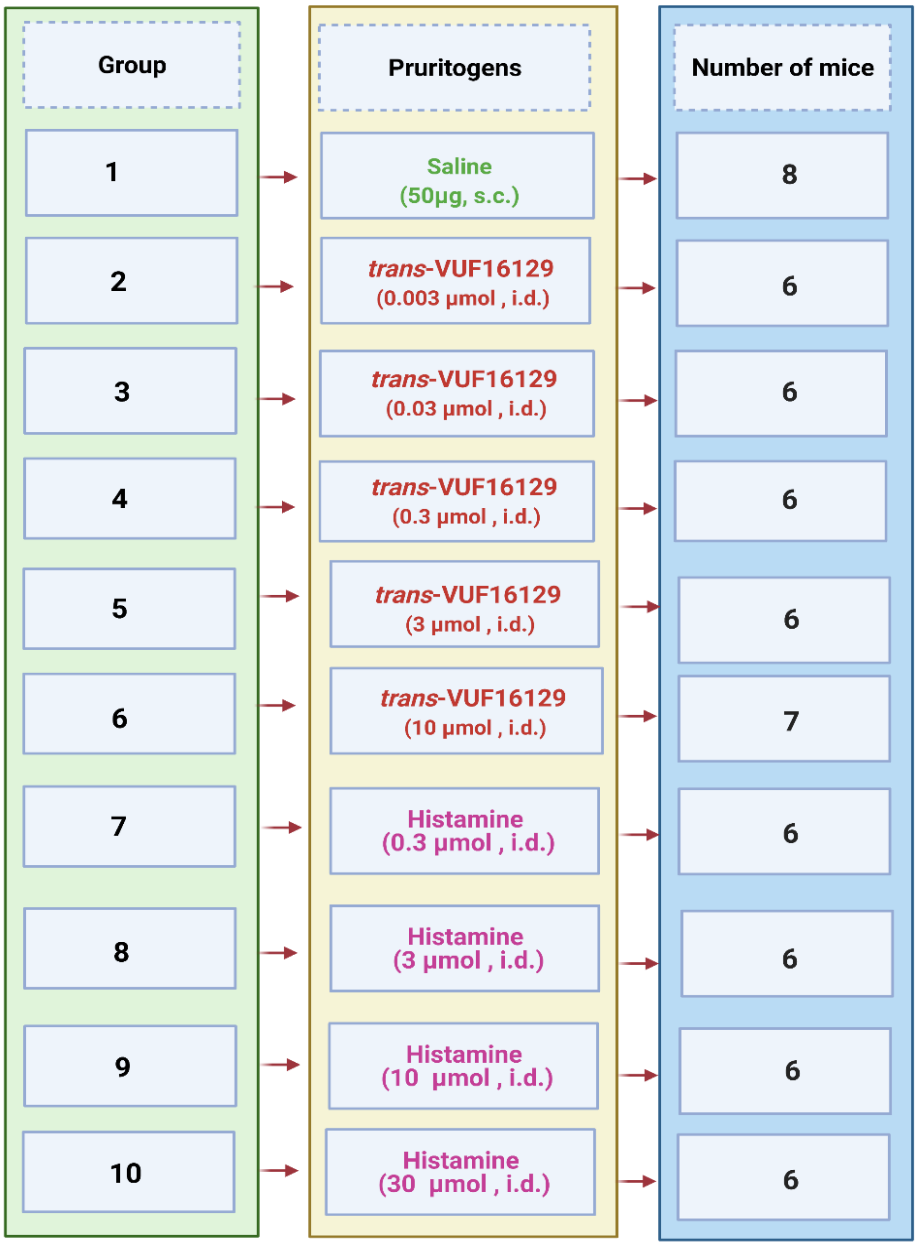
4.2.5 Design of the experiments

4.2.5.1 To determine the role of H_4R in histaminergic itch by using H_4R agonists with photo-switchable activity.

Experiment 1: Induction of itch with H_4R photo-switchable agonist

After 3 consecutive days of habituation mice were randomly assigned into 9 groups as indicated in Table 4.1. On the experimental day (4^{th} day), mice were weighed and habituated to a small plastic chamber for 30 minutes. Then, each mouse received a different dose of *trans*-VUF16129 (0.003-10 µmol, i.d.) into the nape of the mouse neck in a volume of 50µL. Because histamine is known to provoke itching, we used it as a positive control for *trans*-VUF16129. Therefore, group 7-10 (Table 4.1) received different dose of i.d. histamine. Behavioural responses after *trans*-VUF16129 or histamine were recorded with a digital video camera over 40 minutes and then the scratching bouts were analysed. The scratching was quantified as the total number of scratches across 40 minutes at 5 minutes intervals. Each mouse was used only once in one experiment. Figure 4.3 shows the experimental timeline and Table 4.1. shows a summary of doses, sites of injection and number of animals in each experimental group.

Table 4.1 Experimental groups in experiment 1



Itch was induced in mice by injection of pruritogens: *trans*-VUF16129 (H_4R agonist) or histamine. Mice were randomly assigned into 10 groups **(Group 1)** Control group mice received i.d. injection of saline in a volume of 50 µL. **(Groups 2-6)** Mice received different doses of *trans*-VUF16129 (0.003, 0.03, 0.3,3 and 10 µmol, i.d.). **(Groups 7-10)** Mice received different doses of histamine (0.3,3, 10 and 30 µmol, i.d.)

Experiment 2: The effects of JNJ 7777120 and bavisant on trans -VUF16129 induced itch

After 3 consecutive days of habituation mice were randomly assigned into 9 groups as indicated in Table 4.2. On the experimental day (4th day), mice were weighed and habituated to a small plastic chamber for 30 minutes. Then, each mouse received either bavisant (10mg/kg=0.59 µmol, i.p.) or JNJ 7777120 (20mg/kg=1.8 μmol), i.p) 15 minutes

before *trans -VUF16129*. Behavioural responses after bavisant , JNJ 7777120 or vehicles were recorded for 40 minutes and analysed at 5 minutes intervals. Control animals received equivalent vehicle solution without bavisant or JNJ 7777120. The whole experiment was run under red light to protects chemical properties of *trans*-VUF16129. Each mouse was used only once, in one experiment. Figure 4.4 shows the experimental timeline and Table 4.2 shows a summary of doses, site of injection and number of animals in each experimental group.

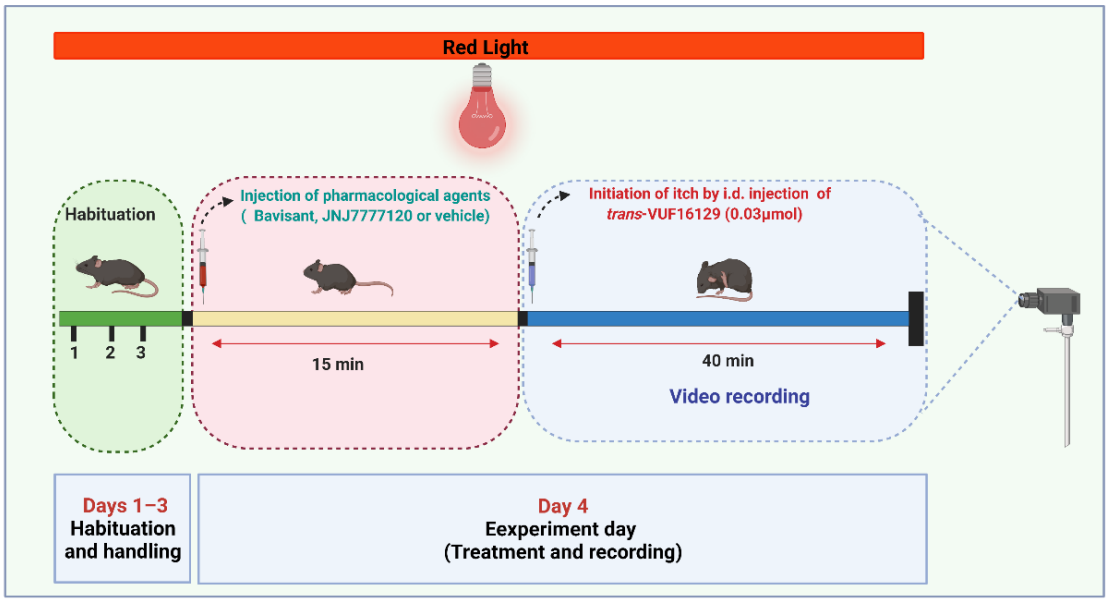
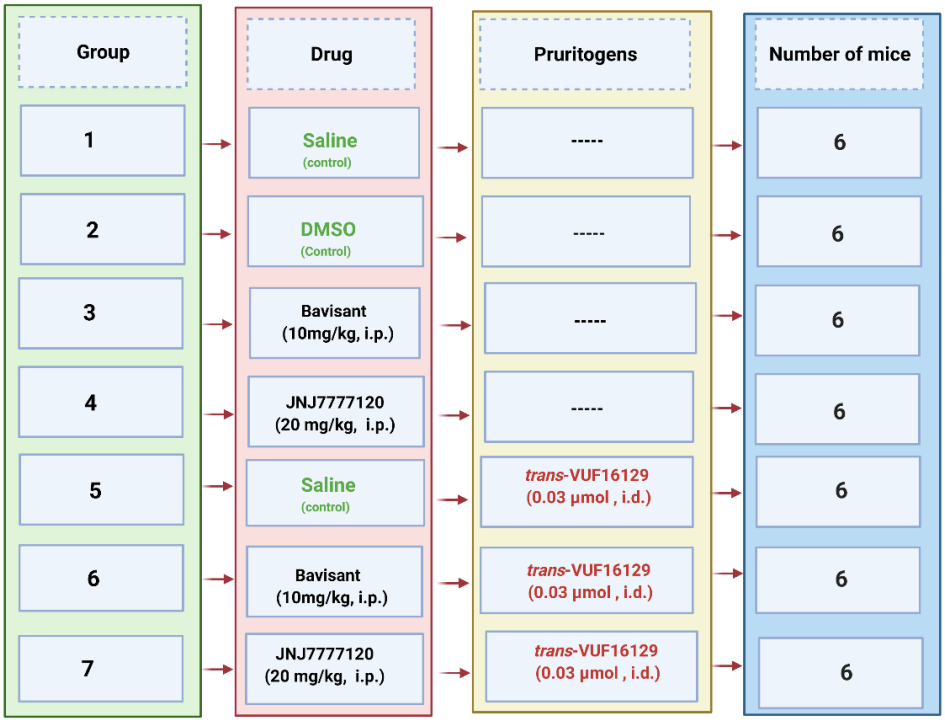


Figure 4.4 Schematic representation of the experimental timeline. The experiment lasted 4 days in total. The green line represents the habituation days (30 minutes) for 3 consecutive days, and the yellow line represent the time after injection of bavisant or JNJ 7777120. The blue line represents the recording time with a digital video camera after induction of itch using *trans*- VUF16129. The above red line indicates that the experiment was conducted under red light.

Table 4.2 Experimental groups in experiment 2



Mice were randomly assigned into 7 groups **(Group 1)** Control group mice received i.p. injection of saline in a volume of 100 µL. **(Group 2)** Control group mice received i.p. injection of DMSO in a volume of 100 µL. **(Group 3)** Mice received i.p injection of bavisant in a volume of 100 µL. **(Group 4)** Mice received i.p. injection of JNJ7777120 in a volume of 100 µL. **(Group 5)** Control group mice received i.p. injection of vehicle in a volume of 100µL 15 minutes prior to i.d. injection of *trans*-VUF16129. **(Group 6)** Mice received i.p. injection of bavisant (10mg/kg, i.p.) in a volume of 100µL 15 minutes before injection of *trans*-VUF16129. **(Group 7)** Mice received i.p. injection of JNJ7777120 (20mg/kg, i.p.) in a volume of 100µL 15 minutes before injection of *trans*-VUF16129.

Experiment 3: The effects of UV light 365 nm on trans-VUF16129-induced itch

After 3 consecutive days of habituation mice were randomly assigned into 9 groups as indicated in Table 4.3. On the experimental day (4th day), mice were weighed and habituated to a small plastic chamber for 30 minutes. Mice were subjected to itch behaviour, induced by injection of *trans*-VUF16129 (0.03 µmole, i.d.) that was prepared as solution and immediately injected into the nape of the mouse neck in a volume of 50 µl. Then, the mice were placed in the experimental box for observation, and we count the number of scratches for 10 minutes to confirm that this compound induced scratching as already determined in previous experiments. After 10 minutes the UV-A light 365 nm was turned on and the behaviour was recorded for another 20 minutes. The total recording time was therefore 30 minutes. The purpose of this experiment was to determine the effect of UV light at 365 nm on the pharmacological activity of *trans*-VUF16129 when changing from active (*trans*) to inactive (*cis*) isoform. The principle of this approach is illustrated in Figure 4.5. Control mice received sterile saline. The whole experiment was run under red light to protects chemical properties of *trans*-VUF16129. Each mouse was used only once, in one experiment. Figure 4.6 shows the experimental timeline and Table 4.3 shows a summary of doses, site of injection and number of animals in each experimental group.

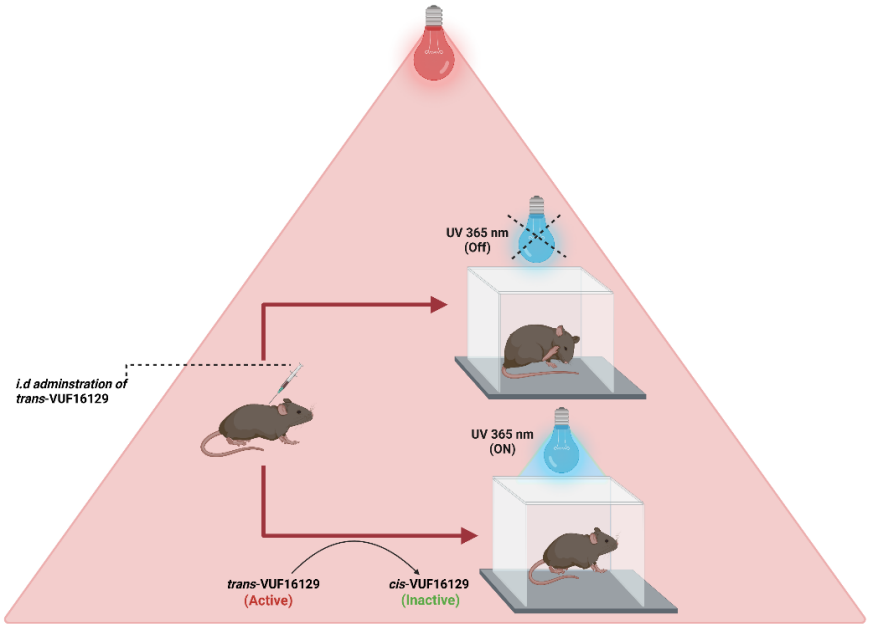
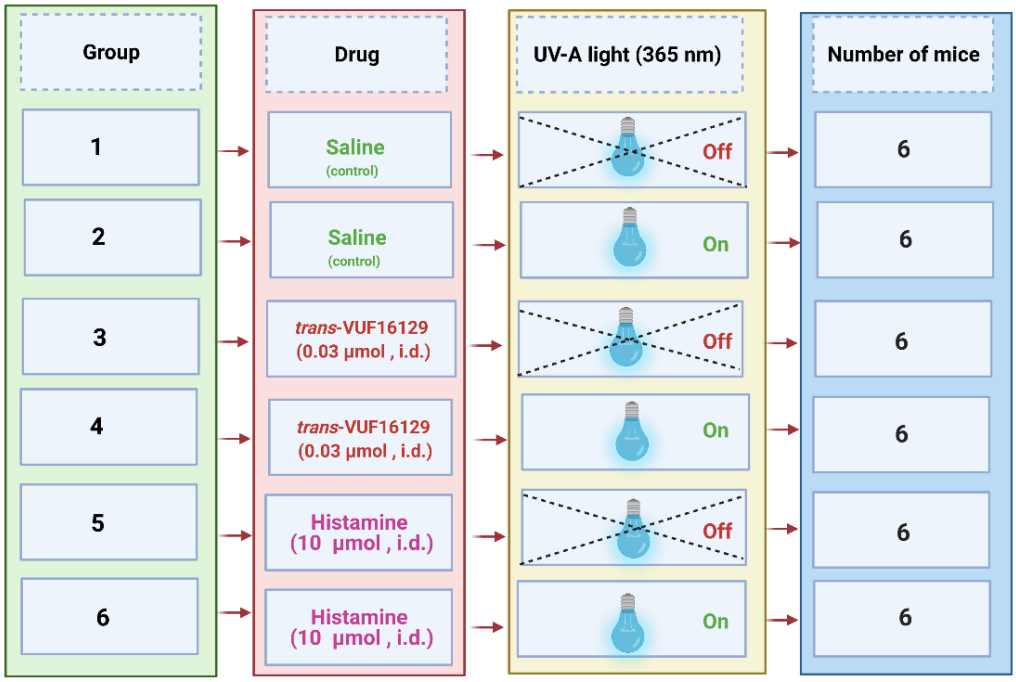


Figure 4.5 Diagram of experimental design of acute itch induction by i.d. injection of photo-switchable ligand, *trans*-VUF16129 into the nape of the mouse neck. Illumination at 365 nm caused pharmacological photo-switching of VUF16129 from *trans* (active, H_4R *agonist*) to *cis* (nearly inactive, weaker H_4R *agonist*) form.

Table 4.3 Experimental groups in experiment 3.



Mice were randomly assigned into 6 groups **(Group 1)** Control group mice received i.d. injection of saline in a volume of 50 µL and UV 365 nm light (OFF). **(Group 2)** Control group mice received i.d. injection of saline in a volume of 50 µL and UV 365 nm light (ON). **(Group 3)** Mice received i.d. injection of *trans*-VUF16129 in a volume of 50 µL and UV 365 nm light (OFF). **(Group 4)** Mice received i.d. injection of *trans*-VUF16129 in a volume of 50 µL and UV 365 nm light (ON). **(Group 5)** Mice received i.d. injection of histamine in a volume of 50 µL and UV 365 nm light (OFF). **(Group 6)** Mice received i.d. injection of histamine in a volume of 50 µL and UV 365 nm light (ON).

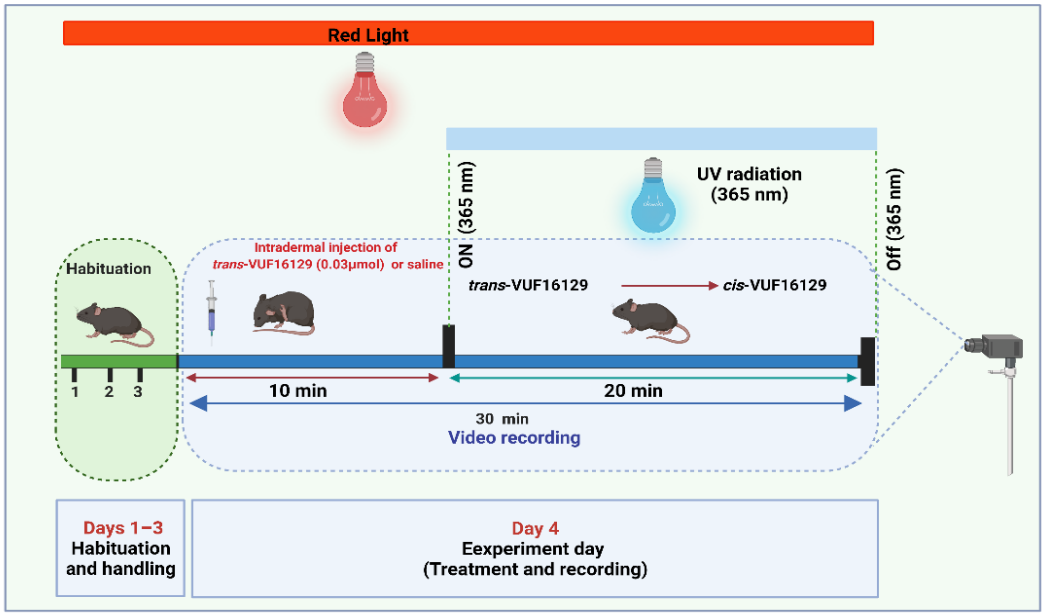


Figure 4.6 Schematic representation of the experimental timeline. The experiment lasted 4 days in total. The green line represents the habituation days (30 minutes) for 3 consecutive days. The blue line represents the recording time with a digital video camera after induction of itch using *trans*- VUF16129. The light blue line represents the time when UV 365nm turned ON and OFF The above red line indicates that the experiment was conducted under red light.

Experiment 4: The effects of cis-VUF16129 on the itch response

After 3 consecutive days of habituation mice were randomly assigned into 9 groups as indicated in Table 4.3. On the experimental day (4th day), mice were weighed and habituated to a small plastic chamber for 30 minutes. In this experiment, mice were injected with i.d. freshly prepared *cis*-VUF16129 at 0.03 µmole into the nape of the mouse neck in a volume of 50 µl. Then, the mice placed in the experimental box and the UV light at 434 nm was turned on for 20 minutes. Immediately after the injection of *cis*-VUF16129, the mice were recorded with a digital video camera over 40- minute where the first 20-minutes was with UV light turned on and the next 20 minutes was with the UV light turned off. The principle of this approach is illustrated in Figure 4.7. Control mice received sterile saline. The whole experiment was run under red light to protects chemical properties of *cis*-VUF16129. Each mouse was used only once, in one experiment. Figure 4.8 shows the experimental timeline and Table 4.3 shows a summary of doses, site of injection and number of animals in each experimental group.

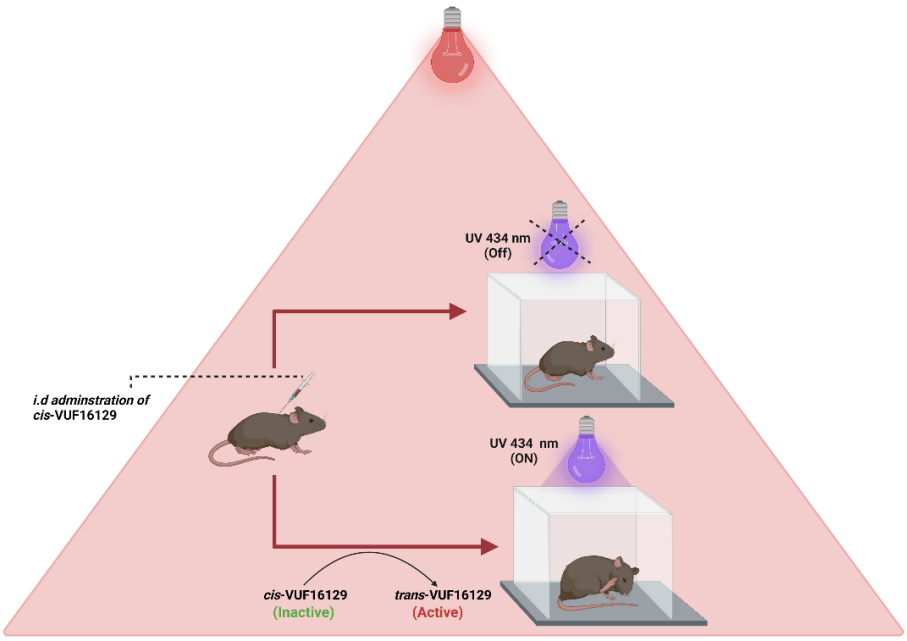


Figure 4.7 Diagram of experimental design of acute itch induction by i.d. injection of photo-switchable ligand, *cis*-VUF16129 into the nape of the mouse neck. Illumination at 434 nm caused pharmacological photo-switching of VUF16129 from *cis* (nearly inactive, weaker H_4R *agonist*) to *trans* (active, H_4R *agonist*) form.

Table 4.3 Experimental groups in experiment 4.

Group	Drug	UV-A light (434nm)	Number of mice
1	Saline (control)	Off	6
2	Saline (control)	On	6
3	*trans*-VUF16129 (0.03 µmol , i.d.)	Off	6
4	*cis*-VUF16129 (0.03 µmol , i.d.)	Off	6
5	*cis*-VUF16129 (0.03 µmol , i.d.)	On	6

Mice were randomly assigned into 5 groups **(Group 1)** Control group, mice received i.d. injection of saline in a volume of 50 µL and UV 434 nm light (OFF). **(Group 2)** Control group mice received i.d. injection of saline in a volume of 50 µL and UV 434 nm light (ON). **(Group 3)** Mice received i.d. injection of *trans*-VUF16129 in a volume of 50 µL and UV 434 nm light (OFF). **(Group 4)** Mice received i.d. injection of *cis*-VUF16129 in a volume of 50 µL and UV 365 nm light (OFF). **(Group 5)** Mice received i.d. injection of *cis*-VUF16129 in a volume of 50 µL and UV 434 nm light (ON).

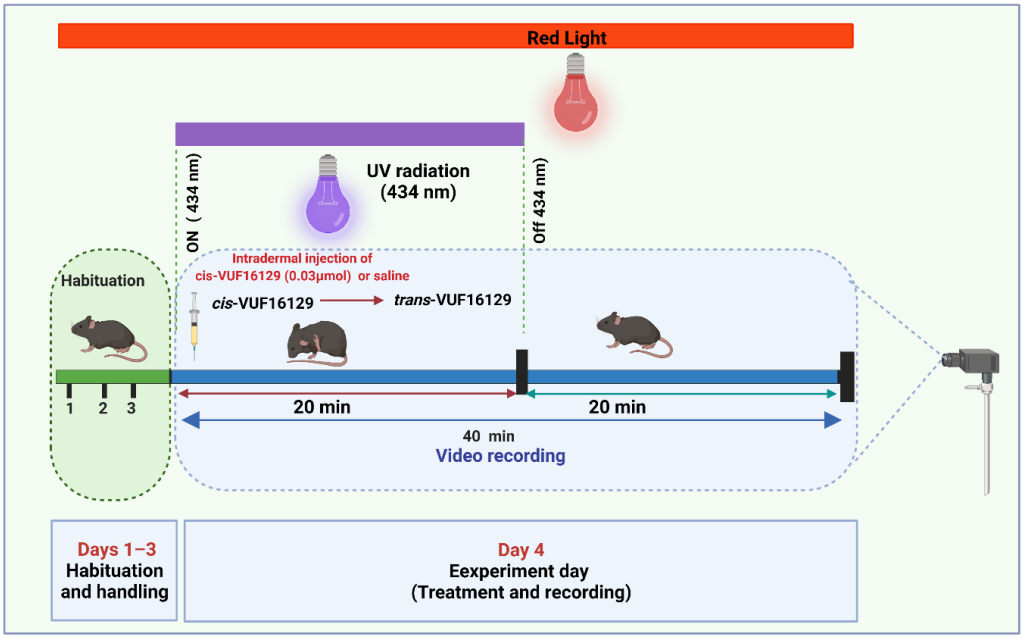


Figure 4.8 Schematic representation of the experimental timeline. The experiment lasted 4 days in total. The green line represents the habituation days (30 minutes) for 3 consecutive days. The blue line represents the recording time with a digital video camera after induction of itch using *cis*- VUF16129. The violet line represents the time when UV 434 nm turned ON and OFF The above red line indicates that the experiment was conducted under red light.

4.3 Statistical analysis

All the data analysis and statistical comparisons were made using GraphPad PrismTM, (version 9.1.2) for Windows/OS (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The results of behavioural changes are presented in the graphs as mean ± SEM. Each group included 6-7 mice. Statistical analysis was performed by one- or two-way analyses of variance (ANOVA) with Bonferroni's multiple comparison post-hoc tests or by unpaired Student's t-test when two groups were compared. A value of $p < 0.05$ *vs* respective control group was considered to be statistically significant.

4.4 Results

4.4.1 *trans*-VUF16129 induced itch in mice.

As illustrated in Figure 4.9 B and C, i.d. injection of *trans*-VUF16129 induced dose-dependent scratching behaviour that lasted for about 40 minutes when compared with the saline control animals. Scratching was not evoked by the lowest dose of *trans*-VUF16129 at 0.003µmol but was consistently observed with higher doses of *trans*-VUF16129, reaching a maximum response at 10µmol. (*trans*-VUF16129 at 10µmol: 128.8±27.8; 3 µmol: 150.3±33.52; 0.3µmol: 150 ±27.6; 0.03µmol: 115.8±11.6 vs. saline: 5.2±1.4, n=6-8). Furthermore, histamine that is known to evoke itching in mice was used as a positive control. Injection of histamine (i.d.) caused scratching in dose-dependent manner. As indicated in Figure 4.9 A and C, scratching was obtained at the lowest doses of histamine at 0.3 μmol and was consistently observed with higher doses at 3 μmol and 10 μmol. However, the highest dose of histamine at 30 μmol did show a decrease in the itch response (histamine at 30 µmol: 68.5±8.6; 10 µmol: 204.4±24.3; 3 µmol: 150.9±12.7; 0.3 µmol:107 ±19.1 vs. saline: 5.2±1.4, n=6-8).

4.4.2 Systemic administration of JNJ 7777120 (H_4R antagonist) , but not bavisant (H_3R antagonist) resulted in a significant inhibition of histaminergic itch induced by *trans*-VUF16129.

As illustrated in Figure 4.10 to determine the role of H_4R in histamine-dependent itch induced by *trans*-VUF16129 in mice, JNJ 7777120, a selective H_4R antagonist was injected i.p.. At the dose of 20 mg/kg (1.8 μmol) JNJ 7777120 reduced itch produced by i.d. administration of 0.03µmol *trans*-VUF16129 as indicated in Figure 4.10 A and C (total number of scratches after treatment with i.p. JNJ7777120 20 mg/kg: 33±9.5 and saline: 115.8±11.6, $P<0.05$, n=6). In contrast, systemic i.p. administration of bavisant, a selective H_3R antagonist, at a dose of 10 mg/kg (0.59 µmol) did not reduce itch produced by *trans*-VUF16129 as indicated in Figure 4.10 B and D (total number of scratches after treatment with i.p. bavisant, 10mg/kg: 130.9 ±29.3 and saline: 115.8±11.6, $P>0.05$, n=6). Given the selectivity of JNJ7777120 and bavisant, these results are suggesting that itch behaviour produced by *trans*-VUF16129 is mediated through H_4R but not H_3R.

4.4.3 *trans*-VUF16129 but not *cis*-VUF16129 produced significant itch in mice.

To evaluate the photo-switching effects of *trans*-VUF16129 *in vivo,* we performed an experiment to show the effect of *trans*-VUF16129 on itch behaviour after i.d. administration under the UV-A light at 365 nm. First, after the i.d. injection of *trans*-VUF16129 the itch response was observed for the first 10 minutes (without any UV-A light but under red light) to make sure that scratching behaviour was induce by *trans*-VUF16129 as already observed in previous experiments. As illustrated in Figure 4.11, after stimulating the photo-switching properties of *trans*-VUF16129 under the effect of UV-A light 365 nm, this light induced the switching (isomerization) of the *trans* form to *cis* isoform and we found that the total number of scratches after i.d. injection of *trans*-VUF16129 when the UV-A light switched on significantly reduced over 20 minutes (the light was continuously turned on).

As indicated in Figure 4.11 A and C, the total number of scratches after local injection of *trans*-VUF16129 (0.03µmol, i.d.) observed within the first 10 minutes (UV-A light turned off) was recorded at 44.4±7.4 (comparing to histamine 48.8±7.2 and saline 1.8±0.7, n = 6). After 10 minutes and confirmation of the presence of itch behaviour, the UV-A light at 365 nm was turned on for another 20 minutes and as indicated in Figure 4.11 B and D the total number of scratches after local injection of *trans*-VUF16129 (0.03µmol, i.d.) was reduced and recorded at 11.5±5 (comparing to histamine 99.8±22 and saline: 1.3±0.6, n=6).

Interestingly, the total number of scratches after local injection of *trans*-VUF16129 (0.03µmol, i.d.) when (UV-A light turned off) was significantly increased compared to group of mice injected with trans-VUF16129 (0.03µmol) (UV-A light turned) : *trans*-VUF16129 (0.03µmol, i.d. UV-A light ON): 11.5±5, *trans*-VUF16129 (0.03µmol, i.d. UV-A light OFF): 58.9±7.8. Table 4.5 below provides a summary of results obtained in experiment 3. Importantly, i.d. administration of histamine was not affected by the illumination with UV-A light suggesting that local photo-isomerization of *trans*-VUF16129 was achieved under UV-A light at 365nm and it weakened the agonistic properties of VUF16129.

In order to further prove the reversible photo-switching properties of VUF16129, after

i.d. injection of *cis*-VUF16129, the total number of scratches was increased when the UV light at 434nm was turned on comparing to the *cis*-VUF16129 without the UV light at 434 nm. Figure 4.12 shows the total number of scratches after local injection of *cis*-VUF16129 (0.03µmol, i.d. UV-A light ON, n=6): 74.28±8.7 and *cis*-VUF16129 (0.03µmol, i.d. UV-A light OFF, n=6): 28.4±4.6. Table 4.6 provides a summary of results obtained in experiment 4. This experiment shows that UV light at 434 nm changed the ligand from *cis* form to trans isoform and this change resulted in alteration of itch behaviour in mice that was aligned with the effect of H_4R agonist on itch response.

Table 4.5 A summary of the results of *trans*-VUF16129 under the effect of the UV light at 365 nm.

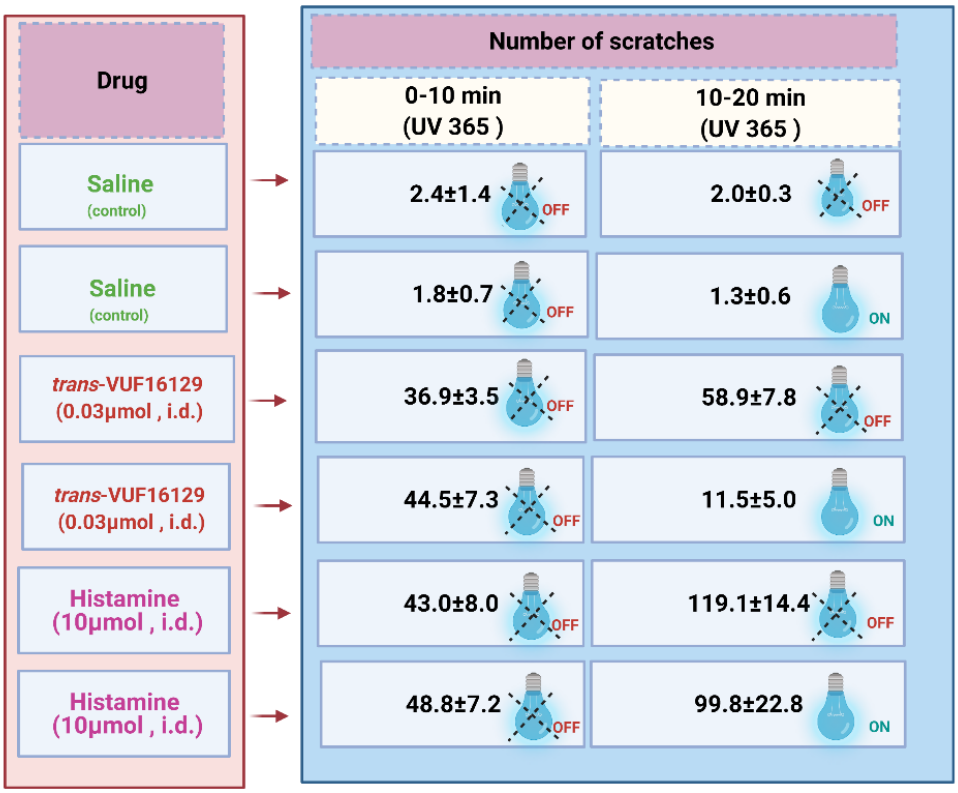
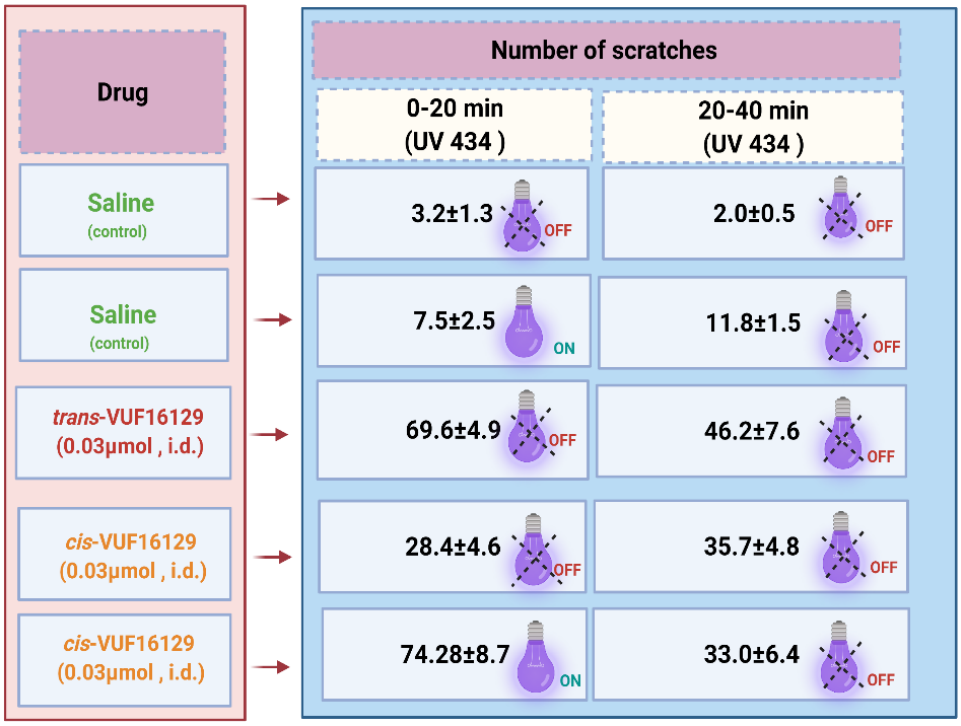


Table 4.6 A summary of the results of *cis*-VUF16129 under the effect of the UV light at 434 nm.



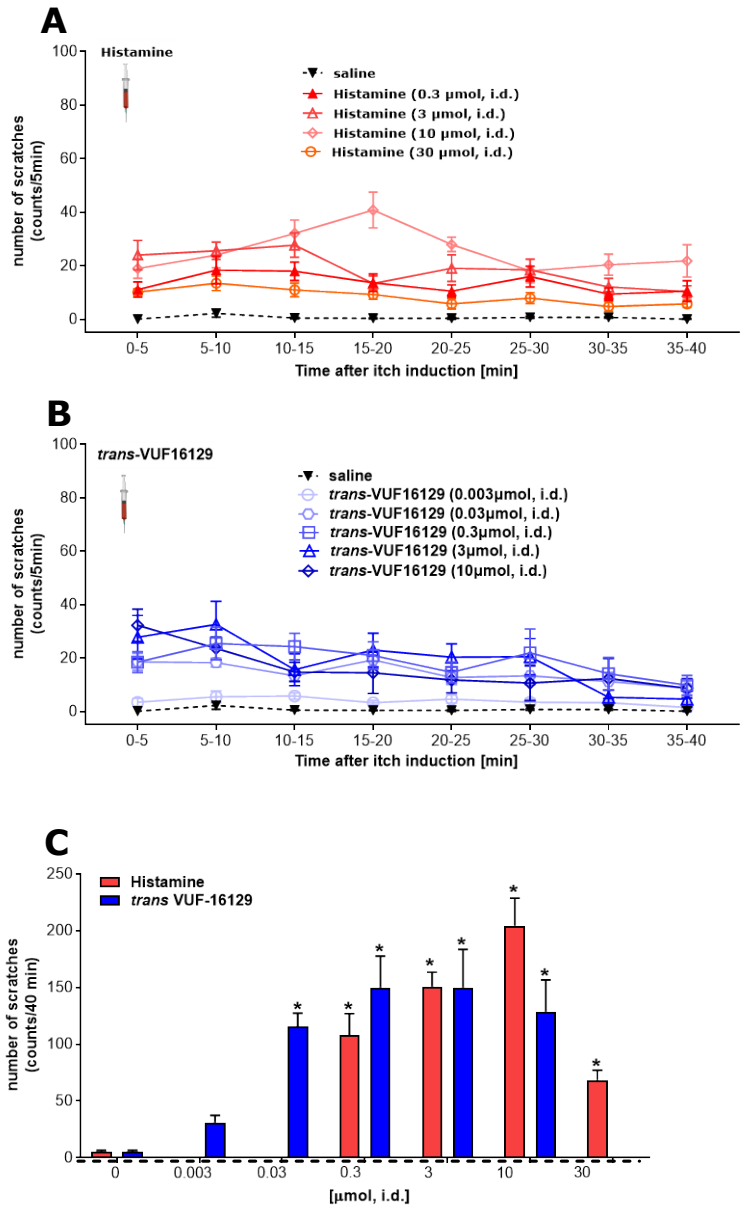


Figure 4.9 local intradermal (i.d) administration of histamine and *trans*- VUF16129 evoked itch behaviour in mice. (A) Time course of scratching behavior induced by i.d. injection of the different doses of histamine in the nape of the neck in mice across a 40-minutes observation period for each treatment. **(B)** Time course of scratching behavior induced by i.d. injection of the different doses of *trans*-VUF16129 in the nape of the neck in mice across a 40-minutes observation period for each treatment. **(C)** Bar graphs showing a total number of scratches across a **4**0 minutes observation period for each treatment. Itch behaviour was recorded, and scratches were counted in 5-minutes intervals for 40 minutes. Dashed line illustrates vehicle induced scratching, Data are presented as mean ± SEM values, n = 6 in each group (*) represents significance vs vehicle control animals; P ≤ 0.05 (A-B, two-way analysis of variance, followed by Bonferroni comparison post hoc test; C- one-way analysis of variance, followed by Bonferroni comparison post hoc test).

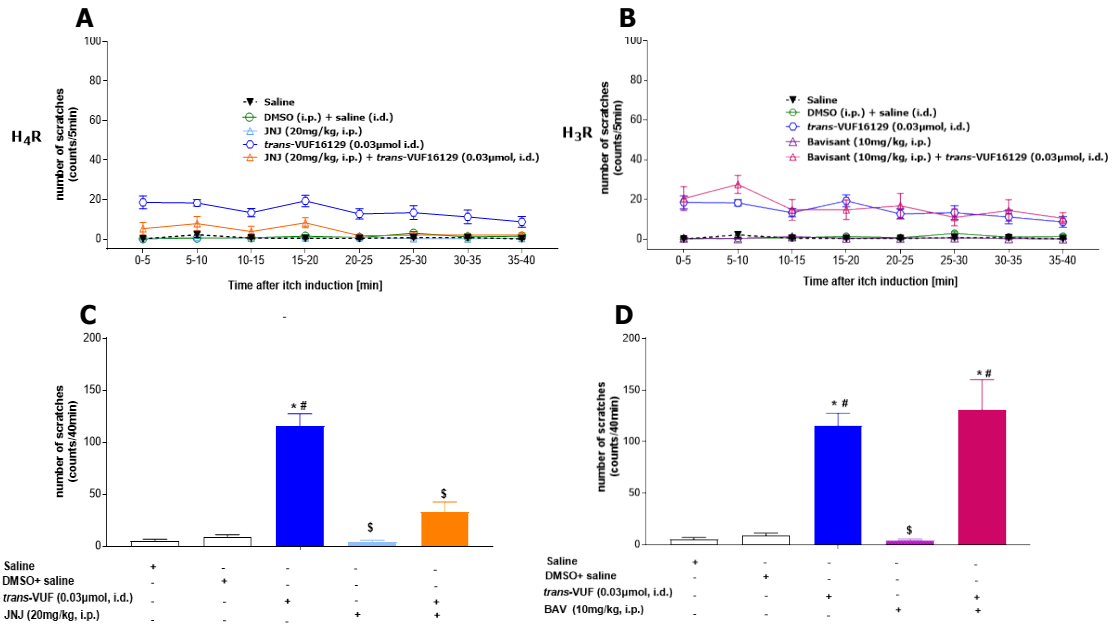


Figure 4.10 Systemic i.p administration of JNJ 7777120 (selective H_4R antagonist) inhibited itch behaviour induced by *trans*-VUF16129, while systemic i.p administration of bavisant (selective H_3R antagonist) did not inhibit itch behaviour induced by *trans*-VUF16129 in mice. (A) Time-course effect of s.c. injection of JNJ 7777120, or vehicle, on the total number of scratches that occurred over a 40 minutes time period after i.d. injection of *trans*- VUF16129 into the nape of the mouse neck. Mice received an i.p. injection of JNJ 7777120 or vehicle 15 minutes before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5 minutes intervals for 40 minutes. (B) Time-course effect of s.c. injection of bavisant , or vehicle, on the total number of scratches that occurred over a 40 minutes time period after i.d. injection of *trans*- VUF16129 into the nape of the mouse neck. Mice received an i.p. injection of bavisant or vehicle 15 minutes before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5 minutes intervals for 40 minutes. (C and D) Bar graphs showing a total number of scratches across a **4**0 minutes observation period for each treatment. Data are presented as mean ± SEM values, n =6 in each group. the number symbol (#) represents significance *vs* mice pre-treated with JNJ 7777120 or bavisant, the asterisk (*) represents significance *vs* vehicle control animals and dollar sign ($)represents significance *vs trans*-VUF16129 ; *P ≤ 0.05, (A-B, two-way analysis of variance, followed by Bonferroni comparison post hoc test; C-D, one-way analysis of variance, followed by Bonferroni comparison post hoc test).

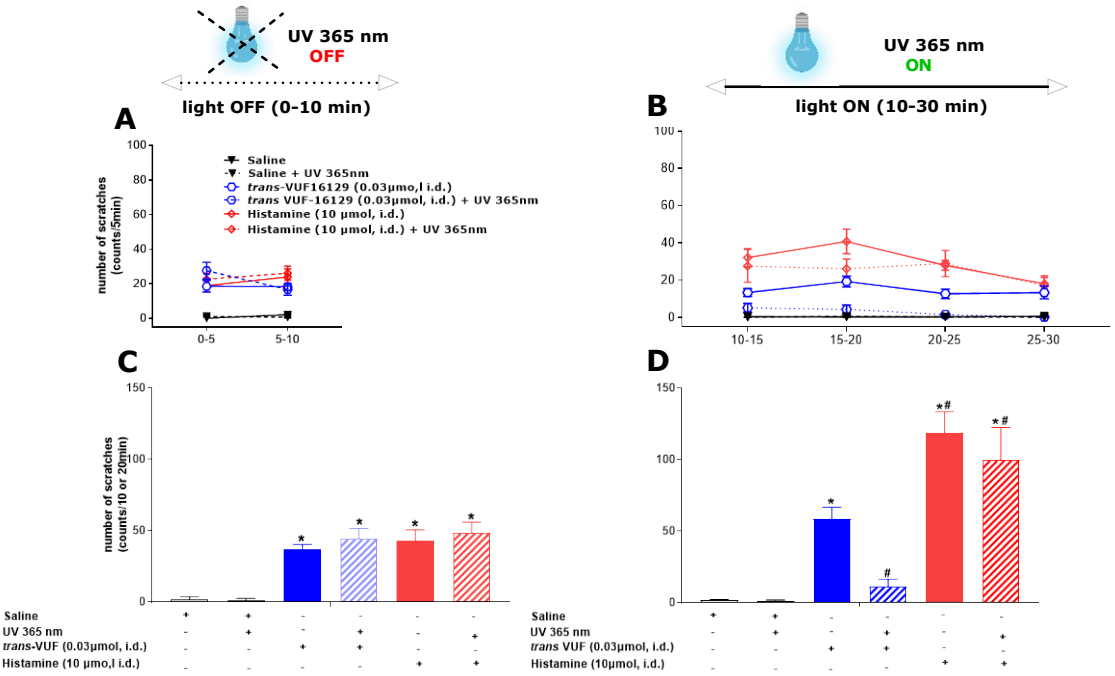


Figure 4.11 UV light (365 nm) switch the VUF16129 from the thermodynamically stable and active agonist *trans* isomer to the *cis* isomer which is inactive form. (A and B) Time-course effect of i.d. injection of *trans*- VUF16129, histamine or vehicle, on the total number of scratches that occurred over a 30 minutes time period after i.d. injection of *trans*- VUF16129 into the nape of the mouse neck. Mice received an i.d. injection of *trans*- VUF16129,Histamine or vehicle 10 minutes before exposed to UV-A light (365 nm). Itch behaviour was recorded, and scratches were counted in 5 minutes intervals for 30 minutes (10 minutes before UV-A light (365 nm) switch ON (A) and 20 minutes after UV-A light (365 nm) switch ON (B) . (C and D) Bar graphs showing a total number of scratches across a 30 minutes observation period for each treatment. Data are presented as mean ± SEM values, n =6 in each group. The number symbol (#) represents significance *vs* mice pre-treated with *trans*-VUF16129 and, the asterisk (*) represents significance vs vehicle control animals; *P ≤ 0.05, (A-B, two-way analysis of variance, followed by Bonferroni comparison post hoc test; C-D, one-way analysis of variance, followed by Bonferroni comparison post hoc test).

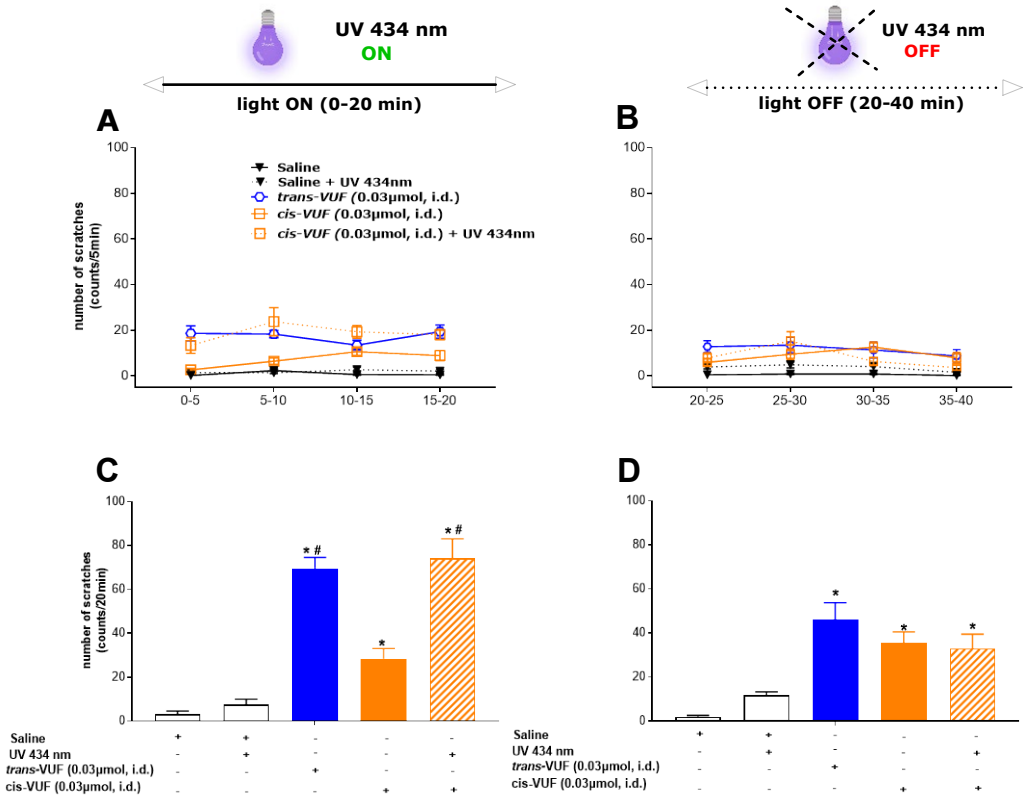


Figure 4.12. UV light (434 nm) switched the VUF16129 from the thermodynamically inactive *cis* isomer to the *trans* isomer which is an active form. (A and B) Time-course effect of i.d. injection of *trans*-VUF16129,*cis*- VUF16129, histamine or vehicle, on the total number of scratches that occurred over a 30 minutes time period after i.d. injection into the nape of the mouse neck. Mice received an i.d. injection of *trans*- VUF16129,*cis*- VUF16129, histamine or vehicle and then the mice will be exposed to UV 434 light for 20 minutes and then turned off the UV 434 light for 20 minutes . Itch behaviour was recorded, and scratches were counted in 5-minutes intervals for 30 minutes (20 minutes when UV light (434 nm) switch ON (A) and 20 minutes when UV light (434 nm) switch OFF (B) . (C and D) Bar graphs showing a total number of scratches across a 40 minutes observation period for each treatment. Data are presented as mean ± SEM values, n =6 in each group. The number symbol (#) represents significance *vs* mice pre-treated with *cis*-VUF16129 and, the asterisk (*) represents significance *vs* vehicle control animals; *P ≤ 0.05, (A-B, two-way analysis of variance, followed by Bonferroni comparison post hoc test; C-D, one-way analysis of variance, followed by Bonferroni comparison post hoc test).

4.7 Discussion

In this chapter, we examined the importance of itch signalling by using a novel and specially designed photo-pharmacology tools allowing to modulate the pharmacological activity of a ligand selectively targeting H_4R. Employment of this tool, allowed us to study the role of H_4R in the mediation of histaminergic itch in mice. This study was first to shown not shown ability of switching pharmacological properties of VUF16129 *in vivo.* We confirmed that VUF16129 showed *in vivo* activity towards H_4R, highlighting the role of H_4Rs in histamine-mediated itch transmission. With this novel approach, researchers may be able to gain a better understanding of the various roles played by H_4R in itch transmission as well as processing of sensory sensations in general.

The discovery of the H_4R opened a new opportunity in the histamine filed. It has been demonstrated that stimulation of H_4Rs regulates the itch response, as pre-treatment with H_4R antagonists decreased the itch induced by both selective H_4R agonists and histamine (Dunford et al., 2007; Marson, 2011). Also, animal studies have demonstrated that treating mice with the H_4R antagonist JNJ7777120 reduces itch caused by i.d. administration of histamine and compound 48/80, with a greater inhibitory effect than seen with H_1R antagonists (Dunford et al., 2007). Furthermore, the observation that i.d. administered a selective H_4R agonist provoked scratching in mice, which was completely inhibited by H_4R antagonist, JNJ7777120, may suggest the presence of H_4R in sensory neurons (Dunford et al., 2007; Rossbach et al., 2011; Desmadryl et al., 2012). Consequently, it appears likely that activation of H_4R results in the excitation of itch-mediating histamine-sensitive afferents by increasing intracellular Ca^{2+} levels. Moreover, the first clinical studies in healthy volunteers by Kollmeier and colleagues, (2014) provided additional evidence that H_4R is involved in the transmission of itch (Kollmeier et al., 2014). In 2015, Murata et al., found that H_4R antagonist significantly reduced itch symptoms in patients with AD (Murata et al., 2015). Despite the evidence that H_4R plays a role in itch, the exact mechanism remains unclear.

Photo-pharmacology allows the pharmacological properties of the drug to be switched on or off, upon irradiation with light of a specific wavelength. Here, it has been demonstrated for the first time that the selective H_4R agonist, *trans*-VUF16129, which was injected locally (i.d.) into the nape of the mouse neck, induced scratching behaviour,

which was associated with the activation of the H_4Rs. The result from this study support previous evidence that local administration of H_4R agonist induced an itch response in mice (Dunford et al., 2007; Shim and Oh, 2008). These findings, as well as our observations, can be supported by the evidence that both mRNA and functional expression of H_4R was detected on epidermal tissues, sensory peripheral neurons, C-afferent fiber terminals and DRG (Dunford *et al.*, 2007; Rossbach *et al.*, 2011; Ohsawa and Hirasawa, 2014; Schneider and Seifert, 2016). In addition, in previous clinical study it has been shown that the expression of H_4R mRNA is higher in keratinocytes of patients suffering from atopic dermatitis comparing to healthy people (Glatzer et al., 2013; Wong et al., 2021), which highlighting the importance of H_4R not only in acute type but also in chronic itch. Several lines of evidence indicate that the H_4R plays a key role in itch sensation. In mast cells, activation of H_4R increases intracellular Ca^{2+} levels, possibly *via* PLC (Hofstra et al., 2003; Shim and Oh, 2008). Furthermore, the presence of H_4R in sensory neurons is supported by the observation that i.d. administration of H_4R agonist evoked scratching in mice, which was totally inhibited by pre-treatment with JNJ7777120, a selective H_4R antagonist (Dunford et al., 2007; Shim and Oh, 2008). Also, similar observations have been reported by Ohsawa and Hirasawa in 2014 as they found that scratching responses in mice are induced by H_4R agonists, which can be reduced by pre-treatment with a selective H_4R antagonist (Ohsawa and Hirasawa, 2014). Similarly, several studies indicated that H_4R knockout showed no itch behaviours compared with wild-type mice (Cowden et al., 2010; Rossbach et al., 2016) further confirming that blocking or inactivating H_4R leads to inhibition of itch sensation.

Interestingly, blocking of H_4R reduced scratching behaviour in models of itch in which usage of H_1R antagonists was either incompletely able to stop the itch response or failed to inhibit allergen-induced scratching behaviour (Rossbach et al., 2009; Yamaura et al., 2009; Davidson and Giesler, 2010). H_4R has dual role in itch including, direct effect on sensory neurons and indirect effect by inhibition of the inflammatory process (Schaper-Gerhardt et al., 2020; Wong et al., 2021). Moreover, studies reported by Thurmond et al. (2008) also support the hypothesis that itch stimuli in the skin lead to stimulation of H_4R expressed in sensory neurons, which subsequently transmit the itch signal from peripheral to the central nervous system (Thurmond *et al.*, 2008). All these evidence, as

well as current observation, support the importance of H_4R in itch progression and modulation, and indicate that H_4R may represent as promising candidate for the treatment of wide range of diseases including acute itch in humans.

To prove that *trans*-VUF16129 induced itch was mediated through H_4Rs, mice were pre-treated with a selective H_4R antagonist JNJ 7777120. This treatment resulted remarkable reduction of scratching response induced by *trans*-VUF16129 confirming VUF16129 selectivity towards H_4R. We also excluded an involvement of H_3Rs using bavisant, a selective H_3R antagonist, which showed no effect on *trans*-VUF16129 evoked scratching. These findings suggest that the scratching behaviour induced by *trans*-VUF16129 was mediated through the activation of H_4R, but importantly not H_3R. The current findings are consistent with the previous reports by Bell et al. (2004) and Dunford et al. (2007) where they found that H_4R but not H_3R is involved in itch and selective targeting of H_4R by using selective antagonists reduce the itch sensation (Bell et al., 2004; Dunford et al., 2007; Yu et al., 2010). Moreover, in a randomized clinical study Kollmeier et al. in 2014 found that using a selective H_4R antagonist inhibited itch induced by histamine (Kollmeier et al., 2014). These findings based on our and earlier previous research strengthen the hypothesis about the involvement of H_4R in peripheral neural transmission of itch.

We also took steps to determine whether the protocol for inducing scratching by *trans*-VUF16129 was suitable as a method for studying itch and the role of H_4R. We, therefore, demonstrated that the scratching in mice was not induced by any of the experimental control procedures such as red light, UV light (343 and 464 nm), saline or DMSO. These control experiments confirmed that the scratching in mice was observed due to the administration of *trans*-VUF16129 to the skin. Our results give the first evidence that upon UV light at 343 nm we could induced *trans–cis* isomerization that was reflected by a decrease of H_4R activity and lead to a remarkable decrease in the number of scratches in animals. Interestingly, UV light at 343 nm did not show any effect on histamine induce scratches which was used as a positive control since the number of scratch episodes induced by histamine did not decrease after exposed to irradiation provided by UV light at 434 nm. In contrast, *cis*-VUF16129 form when exposed to UV light at 434 nm, lead to an increase in the total number of scratches. This trend can be explained by the increase

in the affinity of the compound to H_4R when it is switching from *cis* which has weak H_4R-agonist activity to the trans isomer which has much stronger H_4R-agonist activity. While showing interesting ability of photo-switchable ligands, these results further clearly highlight the critical role of peripheral H_4R in itch signal transmission.

In summary, our findings provide the first *in vivo* evidence to support the importance of peripheral H_4R in the regulation of itch by using photo-pharmacological approaches. Developing biological data using different approaches targeting H_4R has the potential for improving the understanding of pathophysiology and sensory processing mechanisms leading to potential new drugs for the treatment of itch.

Effect of systemic and local peripheral administration of a novel histamine-binding protein Votucalis on neuropathic pain

5.1 Introduction

Neuropathic pain remains a significant clinical problem worldwide. It is affecting 6.9 to 10% of general population (Van Hecke et al., 2014). It has been defined by the International Association for the Study of Pain (IASP) as *"pain arising as a direct consequence of a lesion or disease affecting the somatosensory system"* (Treede et al., 2008; Jensen et al., 2011). The most common symptoms of neuropathic pain range from minor painful sensation such as tingling, prickling and dysesthesia (an unpleasant abnormal sensation, whether spontaneous or evoked) to major symptoms such as allodynia (pain due to a stimulus that does not normally provoke pain), hyperalgesia (increased pain from a stimulus that normally provokes pain), and paraesthesia (abnormal sensations, such as 'pins and needles', tingling, prickling, reduced or even loss of sensation) (Woolf and Mannion, 1999; Bennett, 2001; Haanpää and Treede, 2010).

Despite advances in the understanding of the aetiology and mechanisms leading to the development and regulation of neuropathic pain, only 40% of patients achieve pain control with existing medications (Breivik et al., 2006; Baron et al., 2010). Up to now, no cures or medicine has shown long-term efficacy, safety and tolerability for neuropathic pain conditions (Brooks and Kessler, 2017). Therefore, there is an urgent need for the investigation of new therapeutic strategies in order to improve management of neuropathic pain that will directly increase the therapeutic outcome for individuals suffering from pain. Recent development of novel ligands targeting the histamine system has provided an interesting tool for further investigation of the role of histamine in nociception and validation of histamine receptors as potential targets for therapeutic intervention in neuropathic pain.

Histamine and histamine receptors play an important role in the regulation of a wide variety of physiological and pathological processes, including pain sensation (Haas et al., 2008; Blandina et al., 2012; Lindskog, 2017). Histamine is one of the most potent

inflammatory mediators, being found in both humans and rodents, and it plays a critical role in both peripheral and central sensitization mechanisms (Mobarakeh et al., 2000; Mobarakeh et al., 2002; Rosa and Fantozzi, 2013).The role of histamine in the regulation of pain is varied; it can either be relieving or aggravating of pain depending on many factors such as site of action, type of receptors and concentration of histamine (Tamaddonfard and Rahimi, 2004, Obara et al., 2020). A growing body of evidence indicates that peripheral histamine signaling is critical in the initiation and progression of chronic neuropathic pain, and blocking these signaling pathways can reduce neuropathic pain in animal models (Rosa and Fantozzi, 2013; Obara et al., 2020). In PNS, as a result of tissue injury or damage, histamine is released, where it contributes to the generation of pain hypersensitivity *via* sensitizing peripheral polymodal nociceptors, which results in increased firing rates and generate action potentials in the neurons (Obara et al., 2020).

In neuropathic pain, histamine released by peripheral mast cells has been demonstrated to be critical in the development of hypersensitivity following nerve injury (Obara et al., 2020). This pathological process *initiate*d by macrophage and neutrophil recruitment is controlled by histamine, a potent mast cell chemoattractant (Smith et al., 2007; Zuo et al., 2003; Obara et al., 2020). Moreover, in inflammatory pain conditions, the release of histamine is linked to the increasing release of a wide range of inflammatory mediators such as leukotrienes, cytokines, and chemokines, which lead to an increase in the transmission of pain signals from PNS to CNS (Rosa and Fantozzi, 2013; Thangam et al., 2018; Branco et al., 2018). Together, all these shreds of evidence suggest that histamine may contribute to the pain sensation in various chronic conditions, particularly in neuropathic pain.

HRs, which are members of the GPCR family, are known to mediate histamine-induced responses. Histamine receptors are expressed in both the PNS and CNS (Lindskog, 2017). To date, four different subtypes of histamine receptors have been identified. The pharmacology and signal transduction properties of these receptors are distinct (Panula et al., 2015; Parsons & Ganellin, 2006; Simons & Simons, 2011). Therefore, depending on the histamine receptor subtype to which histamine is bound, it exerts differential effects on neuropathic pain (Obara et al., 2020). Targeting HR with HR antagonist was shown to

treat and control different type of diseases (Thurmond et al., 2008; Pino-Ángeles et al., 2012) . Recently, there is evidence that histamine may play a potential role in pain perception particularly, neuropathic pain through H_3R and H_4R and targeting these types of receptors could help to control chronic pain (Popiolek-Barczyk et al., 2018; Obara et al., 2020). Indeed, histamine and histamine receptors have long been attractive targets for therapeutic uses in conditions where pain is a symptom requiring intervention and treatment (O'Donoghue and Tharp, 2005; Obara et al., 2020).

Targeting histamine receptors is one of the promising targets for the development of novel analgesic agents. In 2018, Popiolek-Barczyk and co-workers demonstrated that targeting H_3R and H_4R using selective receptor antagonists represents a strong anti-nociceptive effect in an animal model of pain. Interestingly, using a combination of H_4R and H_3R shows more analgesic effect than each drug alone (Popiolek-Barczyk et al., 2018). Also, this approach showed analgesic effect more efficient than morphine, which is considered the gold standard in chronic pain treatment (Popiolek-Barczyk et al., 2018). Based on these results, it means that neuropathic pain may be regulated by more than one receptor. Therefore, a compound that targets histamine itself and blocks its effects on all receptors may represent a novel target and potentially more efficient strategy than targeting each receptor alone.

The discovery of histamine-binding proteins in the saliva of ticks, has provided a new and powerful tool to re-examine the role of histamine in the regulation of neuropathic pain (Chmelař et al., 2019; Weston-Davies et al., 2005). Votucalis, as described earlier in Chapter 3 is one of these proteins, that naturally occurs in tick's saliva *Rhipicephalus appendiculatus* its anti-inflammatory activity counters the host's immunological response at the tick-feeding site (Paesen et al, 1999). The mechanism of Votucalis is based on scavenging of endogenous histamine (Paesen et al, 1999; Ryffel et al., 2005). Interestingly, there are several pre-clinical studies showing therapeutic effects of Votucalis through scavenging of endogenous histamine in models of acute respiratory distress syndrome (ARDS), asthma, conjunctivitis and allergic rhinitis (Ryffel et al., 2005; Weston-Davies et al., 2005; Weston-Davies et al., 2006). All these positive results showed potential to investigate the effect of Votucalis in model of chronic pain , in particular neuropathic pain.

The experiments in this Chapter were designed to explore for the first time the analgesic efficacy of Votucalis based on scavenging of endogenous histamine and focused on a mouse model of chronic neuropathic pain.

5.2. Materials and Methods

5.2.1 Subjects

Experiments in this chapter were carried out using adult male C57BL/6J mice (8 weeks of age; 20–25 g; Charles River Laboratories, Harlow, UK) as previously described in Chapter two (Section 2.2). Animals underwent surgery for a model of experimental mononeuropathy, a unilateral chronic constriction injury to the sciatic nerve (CCI; Section 2.5.1) and were behaviourally assessed (Section 2.6) prior to surgery (to obtain baseline values) and post-surgery (from day 6) until (day 10 post surgery) from sham and CCI. All animals were behaviourally assessed (Section 2.6) prior to injection (to obtain baseline values) and post injection. All animals were tested for signs of mechanical allodynia using calibrated von Frey filaments (Section 2.6.2) and for signs of thermal hyperalgesia using the Hargreaves' thermal apparatus (Section 2.6.3). A schematic diagram of this experiment is shown in Figure 3.1.

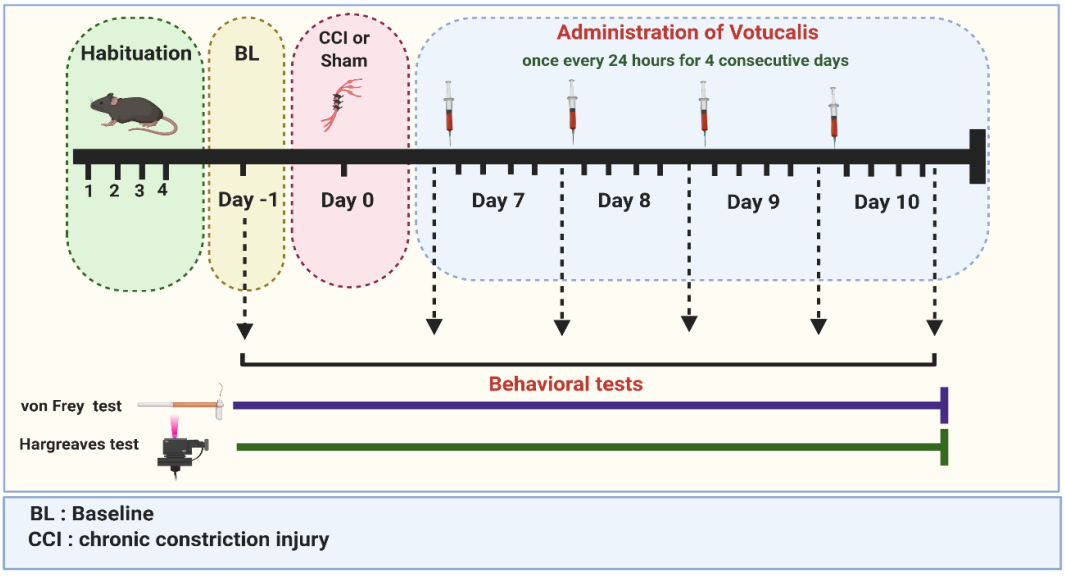


Figure 5.1 A schematic diagram summarising timeline of the experiment. A representation of the experimental schedule in which neuropathic pain (CCI) induction (day 0), Votucalis treatments (days 7, 8, 9 and 10 post CCI) and behavioural assessment for mechanical and thermal stimulation thresholds were taken one day before CCI as baseline (day -1) and then repeated consecutively after administration of Votucalis 30 min, 1,2,4 and 24 hours for a period of 4 days.

5.2.2 Preparation and administration of drugs:

As mentioned in the previous Chapter, Votucalis was provided by Akari Therapeutics Plc (UK) as a stock solution of 5.8 mg/mL in phosphate-buffered saline and was stored at -80C. For all administrations, Votucalis was thawed immediately before injections and prepared in a vehicle (sterile saline; 0.9% NaCl; Fresenius Kabi Ltd., UK) solution at required concentrations as described below.

Systemic intraperitoneal (i.p.) administration. Mice were weighed and randomised to receive either Votucalis or vehicle; they were injected i.p. with Votucalis at 1, 3, 10, 20 and 40 mg/kg or vehicle (saline) solution without Votucalis as a control group. Votucalis/vehicle was injected once daily at the same time every 24 hours in days 7, 8, 9 and 10 post induction of neuropathic pain. The animals received a total of 4 i.p. injections of Votucalis /vehicle.

Peripheral intraplantar (i.pl.) administration. Mice were randomised to receive either Votucalis or vehicle; they were injected i.pl. with Votucalis at 0.0075, 0.025, 0.075 and 0.25 mg per paw or equivalent vehicle (saline) solution without Votucalis as a control group. Injections were given over 1 min in a volume of 50 µL without anaesthesia into the plantar surface of the animal hind paw ipsilateral the sciatic nerve injury. Votucalis/vehicle was injected once daily at the same time every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. The animals received a total of 4 i.pl. injections of Votucalis /vehicle. A drugs were injected in a volume of 100 µl for i.p. administration and of 50µl for and i.pl. administrations.

5.2.3 Neuropathic pain model

5.2.3.1 Induction and assessment of neuropathic pain

The method for the induction, and assessment of neuropathic pain is described in detail in Chapter 2.

5.2.4 Design of the experiments

To prove the anti-nociceptive efficacy of Votucalis after systemic and local peripheral administration two experiment were conducted

Experiment 1: Anti-nociceptive efficacy of systemic administration of Votucalis

In this experiment, CCI mice were divided into 8 different groups as indicated in Table 5.1. Baseline (basal pain threshold) for mechanical and thermal hypersensitivity thresholds were taken one day before CCI and sham surgery on (day -1). Both mechanical and thermal hypersensitivity were measured at the lateral plantar surface of the hind paw. Then the behavioural tests were repeated on day 7 post surgery (CCI and sham) and continued for 3 consecutive days on day 8, 9 and 10. Both tests were performed prior the administration of Votucalis i.p. and were repeated at 30 min, 1, 2, 4, 6, 8 and 24 hours after each of Votucalis administrations across four test days. Each animal first underwent von Frey testing followed by the Hargreaves test. Votucalis/vehicle was injected once daily at the same time every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. Sham control mice were divided into 2 groups (group 7 and 8) as indicated in Table 5.1. The behavioural tests and treatment schedule were done as described in CCI group.

Table 5.1 Experimental groups in experiment 1

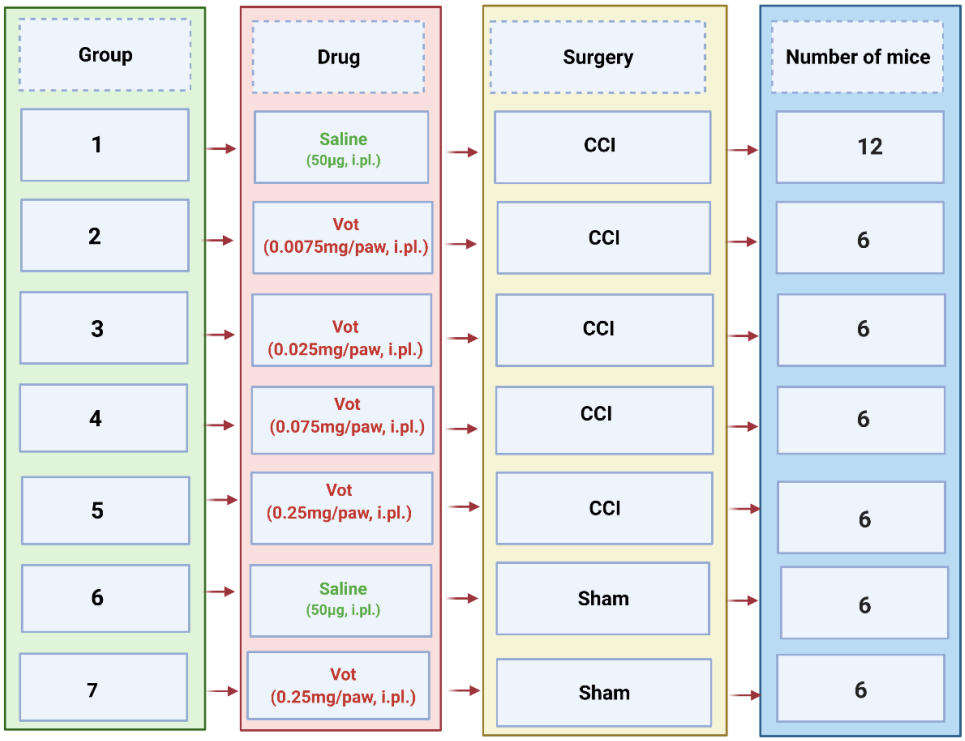
CCI: Chronic Constriction Injury, **Vot:** Votucalis, **i.p.:** intraperitoneal administration.
CCI and sham mice were randomly assigned into 8 groups (n=6-12), received different doses of Votucalis /vehicle (saline) control as following **(Group 1)** CCI mice received i.p. injection of vehicle (saline) in a volume of 100 µL ; **(Groups 2-6)** CCI mice received i.p. injection of different doses of Votucalis (1,3,10,20, and 40mg/kg); **(Group 7)** Sham mice received i.p. injection of vehicle (saline) in a volume of 100 µL; **(Group 8)** Sham mice received i.p. injection of Votucalis (40mg/kg).

Experiment 2: Anti-nociceptive efficacy of local peripheral administration of Votucalis

In this experiment CCI mice were divided into 5 different groups as indicated in Table 5.2. Baseline (basal pain threshold) for mechanical and thermal hypersensitivity thresholds were taken one day before CCI and sham surgery on (day -1). After that, the behavioural tests were repeated on day 7 post surgery (CCI and sham) and continued for 3 consecutive days on day 8, 9 and 10. Both tests were performed prior to the localised peripheral administration of Votucalis (i.pl.) and were repeated at 30 min, 1, 2, 4, 6, 8 and 24 hours after each Votucalis administrations across four test days. Each animal first underwent von Frey testing followed by the Hargreaves test. Votucalis/vehicle was injected once daily at the same time every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. Sham control mice were divided into 2

groups (group 6 and 7) as indicated in Table 5.2. The behavioral tests and treatment schedule were done as described in CCI group.

Table 5.2 Experimental groups in experiment 2



CCI: Chronic Constriction Injury, **Vot :** Votucalis, **i.pl.:** intraplantar administration
CCI and sham mice were randomly assigned into 7 groups (n=6-12), received different doses of Votucalis /vehicle(saline) control as following **(Group 1)** CCI mice received i.pl. injection of vehicle (saline) in a volume of 50 µL; **(Groups 2-5)** CCI mice received local i.pl. injection of different doses of Votucalis (0.0075,0.025,0.075,and 0.25mg/paw); **(Group 6)** Sham mice received i.pl. injection of vehicle(saline) in a volume of 50 µL; **(Group 7)** Sham mice received i.pl. injection of Votucalis (0.25mg/paw).

5.3 Statistical analysis

All the data analysis and statistical comparisons were made using GraphPad PrismTM, (version 9.1.2) for Windows/OS (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The results of behavioural changes are presented in the graphs as mean ± SEM. Each group included 6-17 mice. Statistical analysis was performed by one- or two-way analyses of variance (ANOVA) with Bonferroni's multiple comparison post-hoc tests or by unpaired Student's t-test when two groups were compared. Repeated measures ANOVA used where drug effects were quantified repeatedly over a period of time. A value of $p < 0.05$ *vs* respective control group was considered to be statistically significant.

5.4 Results:

5.4.1 CCI induced neuropathic pain in mice

CCI procedure led to the development of both mechanical allodynia and thermal hyperalgesia on the mid-plantar paw area supplied by the sciatic nerve as demonstrated in Figure 5.1. Neuropathic sensitisation of behavioural reflexes, characteristic of the CCI model, were assessed at 6-7 days after the surgery. As illustrated in Figure 5.2 (A and E), the development of behavioural signs of neuropathic pain on the ipsilateral side after the nerve injury were observed as mechanical allodynia ($t_{(61)}$=27.61, P<0.0001, A), and thermal hyperalgesia ($t_{(35)}$=12.69, P<0.0001, E). Indeed, no change in mechanical or thermal hypersensitivity from baseline in contralateral sides of CCI mice were observed (Figure 5.2 B and F). Moreover, mice that underwent sham surgery (controls), showed no change from baseline threshold in both ipsi- and contralateral sides in mechanical (Figure 5.2 C and D) or thermal hypersensitivity (Figure 5.2 G-H). Apart from these changes, post-operative animals appeared healthy, had no signs of weight loss and were handled without any distress being evident. Also, during this study none of the animals developed any signs of autotomy.

5.4.2 Systemic and peripheral administrations of Votucalis significantly attenuated mechanical hypersensitivity in neuropathic mice

The analgesic effectiveness of systemic (i.p) administration of Votucalis was assessed by measuring the paw withdrawal threshold in response to mechanical stimuli using von Frey filaments in mice with neuropathic pain (CCI model) after 7 days of surgery. After systemic administration the mechanical hypersensitivity in CCI mice was significantly reduced with the three highest doses of Votucalis (10, 20 and 40 mg/kg), compared to vehicle controls alone (Figure 5.3 A, C); effect of the drug: $F_{(5, 812)}$ = 102.2, P<0.0001). The area under the curve (AUC) summarizing time-course effect of i.p. injection of Votucalis and or vehicle on the mechanical withdrawal threshold measured with the von Frey filaments: after i.p. administration of Votucalis 10 mg/kg: 15.2 ±1.4 ,20 mg/kg: 18.3 ±0.8, 40 mg/kg: 21.3 ±0.9, and vehicle: 2.7±0.3).

The highest reduction in mechanical hypersensitivity was after 1-2 hours of the first injection of Votucalis (10, 20 and 40 mg/kg) when compared with vehicle controls and this effect was maintained at 4 hours after the administration. However, 24 hours after

the administration of Votucalis the effect was no longer observed. A similar pattern of analgesic effect was observed after each of four consecutives daily systemic administration of Votucalis (10, 20 and 40mg/kg; Figure 5.2) potentially suggesting lack of pharmacological tolerance to Votucalis. No significant difference in mechanical hypersensitivity was observed with the lowest dose of Votucalis (1 and 3 mg/kg) when compared to saline control group (AUC after i.p. administration of Votucalis 1 mg/kg: 3.9±0.4 ,3 mg/kg: 5.1 ±0.5, and vehicle: 2.7±0.3; Figure 5.3. A and C; $P>0.05$, n=12-6).

After peripheral (i.pl.) administration of Votucalis the mechanical hypersensitivity in CCI mice was significantly reduced with the three highest dose of Votucalis, 0.025, 0.075 and 0.25 mg/paw, when compared to vehicle controls alone (Figure 5.3 B and D, $P<0.0001$). AUC summarizing time-course effect of i.p. injection of Votucalis and or vehicle on the mechanical withdrawal threshold measured with the von Frey filaments: after i.pl. administration of Votucalis 0.025 mg/paw: 10.1 ±0.6 ,0.075 mg/paw: 13.8 ±0.7, 0.25 mg/paw: 21.3 ±1.1, and vehicle: 3.8±0.2.

The greatest reduction in mechanical hypersensitivity was observed 4 hours after the first injection of Votucalis (0.025, 0.075 and 0.25 mg/paw), when compared with saline controls and this effect gradually declined. Again, after 24 hours of Votucalis administration the effect was no longer observed (Figure 5.3 B). A similar pattern of analgesic effect was observed after each of the four consecutive daily i.pl. administrations of Votucalis (0.025, 0.075 and 0.25 mg/paw), that may potentially suggest a lack of pharmacological tolerance after peripheral administration of Votucalis. No significant difference in mechanical hypersensitivity was seen with the lowest dose of Votucalis (0.0075 mg/paw) when compared to the saline control group (AUC after i.pl. administration of Votucalis 0.007 mg/paw: 6.1±0.5 and vehicle: 2.7±0.3; Figure 5.3. B and D; $P>0.05$, n=6-12).

5.4.3 Systemic but not peripheral administration of Votucalis produced a weak effect on thermal hypersensitivity in neuropathic mice

The analgesic efficacy of systemic (i.p.) and localised peripheral (i.pl.) administration of Votucalis was also assessed by measuring the paw withdrawal latency in response to thermal stimuli using the Hargreaves test in CCI mice. Systemic administration of

Votucalis produced a weak effect on heat hypersensitivity in comparison with control vehicle group (Figure 5.4 A and C; P=0.03). However, no significant difference in heat hypersensitivity was observed at any doses of Votucalis tested after localised peripheral administration (Figure 5.4 B and D P=0.34, n=6-12). AUC summarizing time-course effect of i.p. injection of Votucalis and/or vehicle on the thermal withdrawal threshold: after i.p. administration of Votucalis 1 mg/ kg: 63.0 ±1.5 ,3 mg/ kg: 62.6 ±1.7, 10 mg/kg: 79.1 ±5.4, 20 mg/kg: 65.1 ±2.1, 40 mg/kg: 71.2±2.5 and vehicle: 69.1±6.3; and after i.pl. administration of Votucalis 0.0075 mg/paw: 70.3±3.6, 0.025 mg/paw: 70.9 ±2.9 ,0.075 mg/paw: 72.2±2.7, 0.25 mg/paw: 73.2 ±2.9, and vehicle: 65.2±2.0)

5.4.4 Systemic and peripheral administration of Votucalis did not significantly attenuate mechanical hypersensitivity in sham controls

The effectiveness of systemic (i.p.) and peripheral (i.pl.) administration of Votucalis was assessed by measuring the paw withdrawal threshold in response to mechanical stimuli using von Frey filaments in sham control-mice 7 days after surgery. Both routes of administration i.p. and i.pl. of Votucalis (40mg/kg or 0.25mg/paw) showed a lack of changes in paw withdrawal threshold in ipsilateral paws (AUC after i.p. administration of Votucalis 40mg/kg: 33.5±1.2 and vehicle: 31.9±1.0; and after i.pl. administration of Votucalis0.25 mg/paw: 32.2 ±1.1 and vehicle: 30.2±0.9, Figure 5.5 Cand D; P>0.05, n=8-6).

5.4.5 Systemic and peripheral administration of Votucalis did not significantly attenuate thermal hypersensitivity in sham controls

The effectiveness of systemic (i.p.) and peripheral (i.pl.) administration of Votucalis was assessed by measuring the paw withdrawal latency in response to thermal stimuli using the Hargreaves test in sham control mice 7 days after surgery. No change in paw withdrawal latency to heat stimuli was observed in sham mice after either i.p. or i.pl. administration of Votucalis (40mg/kg or 0.25mg/paw) in ipsilateral paws (AUC after i.p. administration of Votucalis 40mg/kg: 151.5±1.8 and vehicle: 146.8±1.7; and after i.pl. administration of Votucalis0.25 mg/paw: 151.4 ±1.7 and vehicle: 147.6±1.9, Figure 5.6 C and D; P>0.05, n=8-6).

5.4.6 Peripheral administration of Votucalis did not significantly change mechanical and thermal hypersensitivity in contralateral (uninjured) paw in both neuropathic and sham mice

As illustrated in Figures 5.7 and 5.8, the contralateral hind paws (uninjured) did not exhibit statistically significant changes of withdrawal threshold for both mechanical and thermal hypersensitivity when compared with the threshold before surgery. The analgesic efficacy of localised peripheral (i.pl.) administration of Votucalis was assessed by measuring the paw withdrawal threshold in response to mechanical stimuli using von Frey filaments. AUC summarizing time-course effect of i.pl. injection of Votucalis and or vehicle on the contralateral paw on the thermal withdrawal threshold: after i.pl. administration in CCI mice of Votucalis 0.025 mg/ paw: 48.8±1.1 ,0.075 mg/ paw: 49.8 ±1.1, 0.25 mg/paw: 50.9 ±1.1, and vehicle: 47.5±1.07, and after i.pl. administration in sham control of Votucalis 0.25 mg/paw: 36.3±0.7, and vehicle: 32.3±1.1, Figures 5.7 C D and E ; $P>0.05$, n=6). Also, no significant difference in thermal hypersensitivity was observed at any doses of Votucalis tested after localised peripheral administration in both CCI and sham control mice. (AUC after i.pl. administration in CCI mice of Votucalis 0.025 mg/paw: 157.7±3.3 ,0.075 mg/paw:163.4 ±3.8, 0.25 mg/paw: 162.8 ±3.4, and vehicle: 161.8±2.3); and after i.pl. administration in sham control of Votucalis 0.25 mg/paw: 152±1.8, and vehicle: 149.1±1.9, Figures 5.8 C ,D and E ; $P>0.05$, n=6)

5.4.7 Peripheral administration of Votucalis produced anti-nociceptive effects at a lower dose range compared to systemic administration.

As summarized in Figure 5.9, Votucalis produced dose-dependent anti-nociceptive effects on mechanical hypersensitivity resulting from injury of the sciatic nerve and subsequent development of neuropathic pain. Overall, localised peripheral (i.pl.) administration of Votucalis produced anti-nociceptive effects at a lower dose range in comparison to systemic (i.p.) administration indicating higher potency after localised peripheral Votucalis administrations observed as a left-ward shift of the dose-response curve for Votucalis.

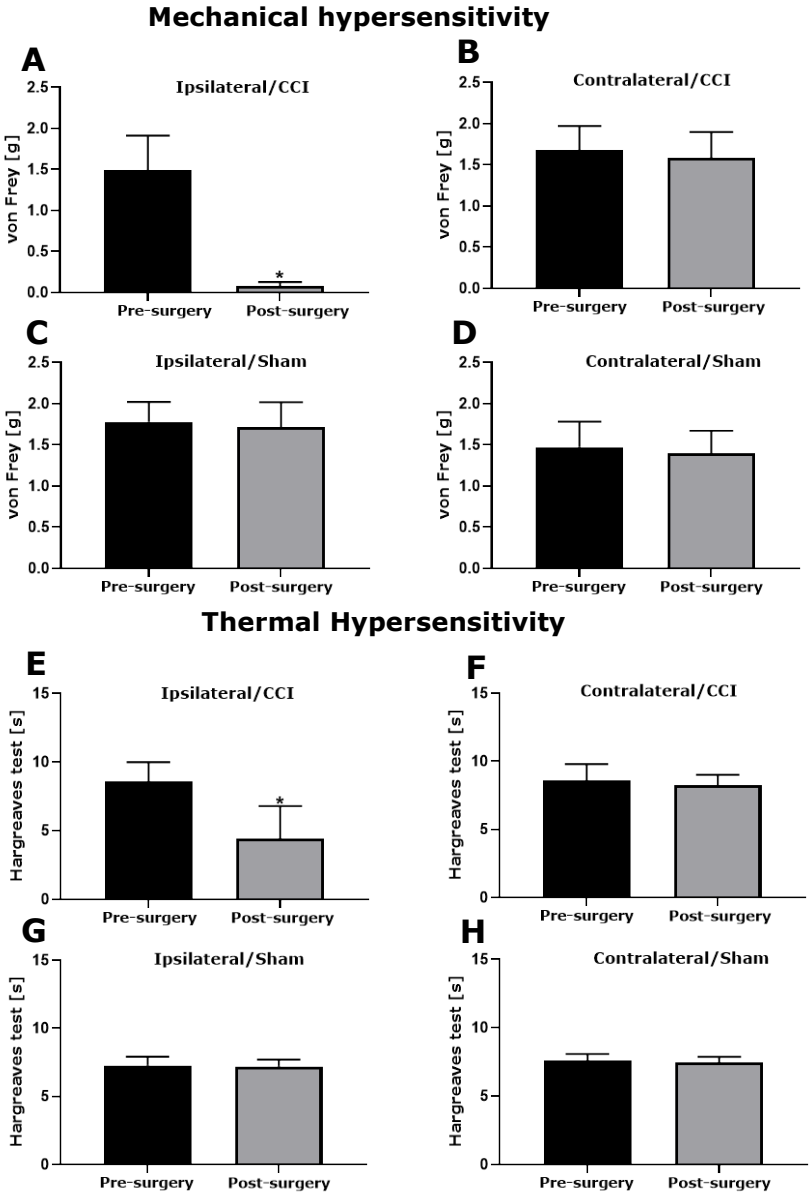


Figure 5.2 Chronic constriction injury (CCI) produced significant change in response to mechanical and thermal stimuli in mice. The von Frey and Hargreaves tests were used to measure the mechanical paw withdrawal threshold and thermal paw withdrawal latency in pre-CCI and post-CCI mice. (A and E) On day 7 after CCI surgery, mice showed an enhanced response to mechanical and thermal stimuli only in the ipsilateral part (injured hind paw) compared with their values before the surgery. Experiment II: On day 7 after sham surgery, mice did not show any different response to mechanical (C) and thermal (G) stimuli in the lateral part of the injured hind paw (ipsi) compared with their values before the surgery. The non-injured hind paw (contra) did not show any significant response in both CCI and sham to mechanical (B and D, respectively) and thermal (F and H respectively) stimulation compared with their values prior to the surgery. Data are presented as mean ± SEM, n = 6-7 in each group, *P<0.05 (paired Student's t-test).

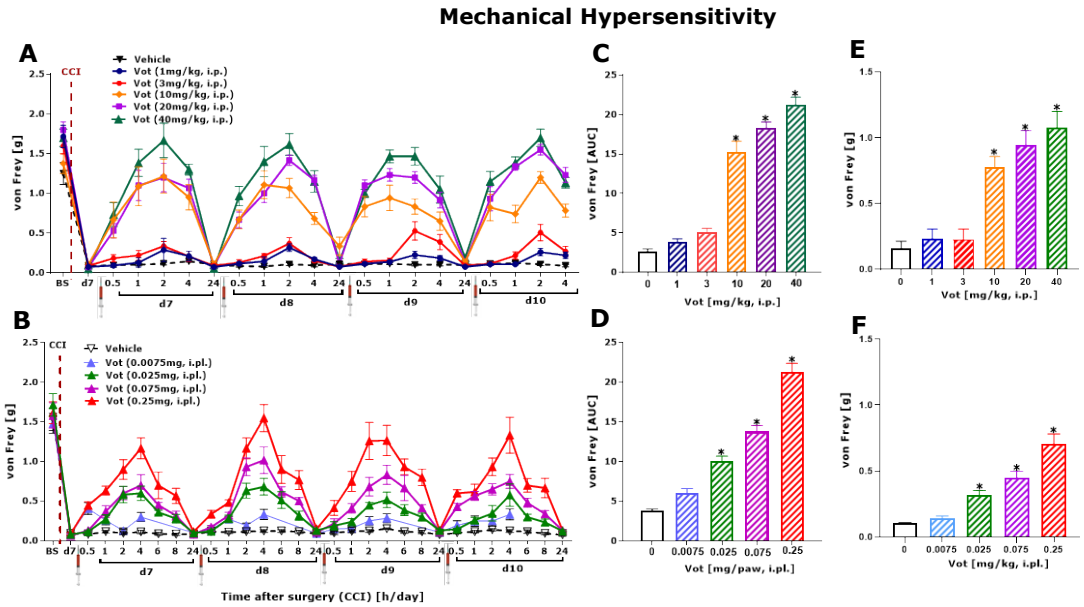


Figure 5.3 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of Votucalis significantly attenuated mechanical hypersensitivity in the chronic constriction injury (CCI) model of neuropathic pain in mice. (A,B) Time-course effect of systemic i.p. injection of Votucalis (Vot, 1-40 mg/kg, A) and i.pl. injection of Votucalis (Vot, 0.0075-0.25 mg/paw, B) administered into the plantar surface of the injured paw, or vehicle, on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5-24 hours after each of four consecutives i.p. **(A)** or i.pl. **(B)** injections. The syringe icon represents each of four i.p. or i.pl. injections of Votucalis. Data are presented as means ± S.E.M, n=6-14 in each group. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. (E) and (F) Comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test. The asterisk (*) denotes significance *vs.* vehicle control animals; *p<0.05 (one-way ANOVA, followed by Bonferroni's comparison post-hoc test, E and F, repeated measure ANOVA followed by Bonferroni's test).

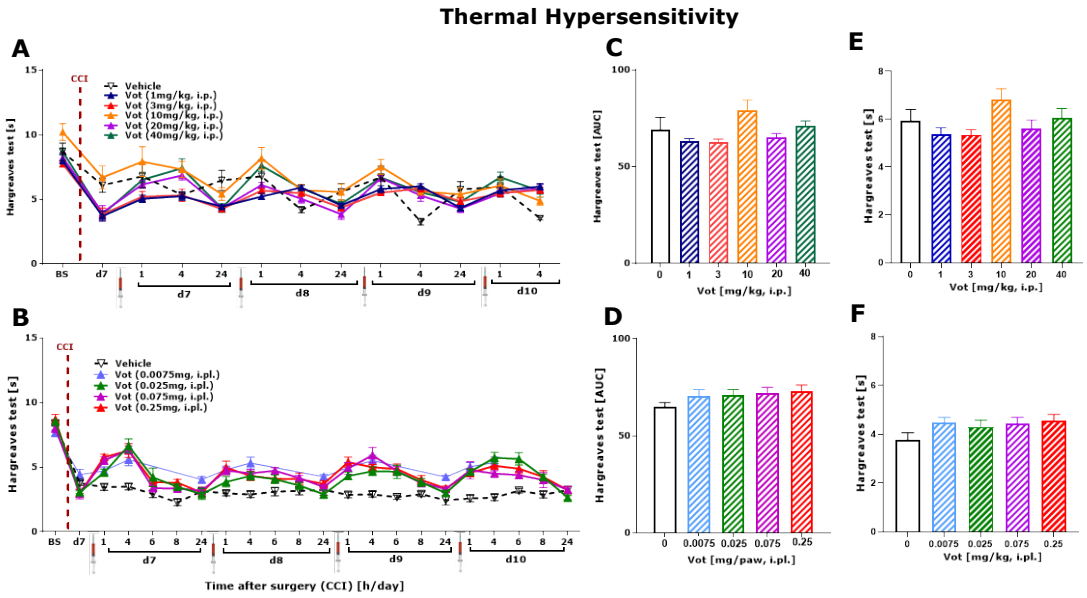


Figure 5.4 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of Votucalis did not attenuate thermal hypersensitivity in the chronic constriction injury (CCI) model of neuropathic pain in mice. (A, B) Time-course effect of i.p. injection of Votucalis (Vot, 1-40 mg/kg, (A) and i.pl. injection of Votucalis (Vot, 0.0075-0.25 mg/paw, (B) administered into the plantar surface of the injured paw, or vehicle (saline), on the thermal withdrawal threshold measured with the Hargreaves test. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5-24 hours after each of four consecutives i.p. (A) or i.pl. (B) injections. The syringe icon represents each of four i.p. (A) or i.pl. (B) injections of Votucalis. Data are presented as means ± S.E.M, n=6-14 in each group. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. (E) and (F) Comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test. Lack of significant changes *vs.* vehicle control animals . (one-way ANOVA, followed by Bonferroni's comparison post-hoc test, E and F, repeated measure ANOVA followed by Bonferroni's test).

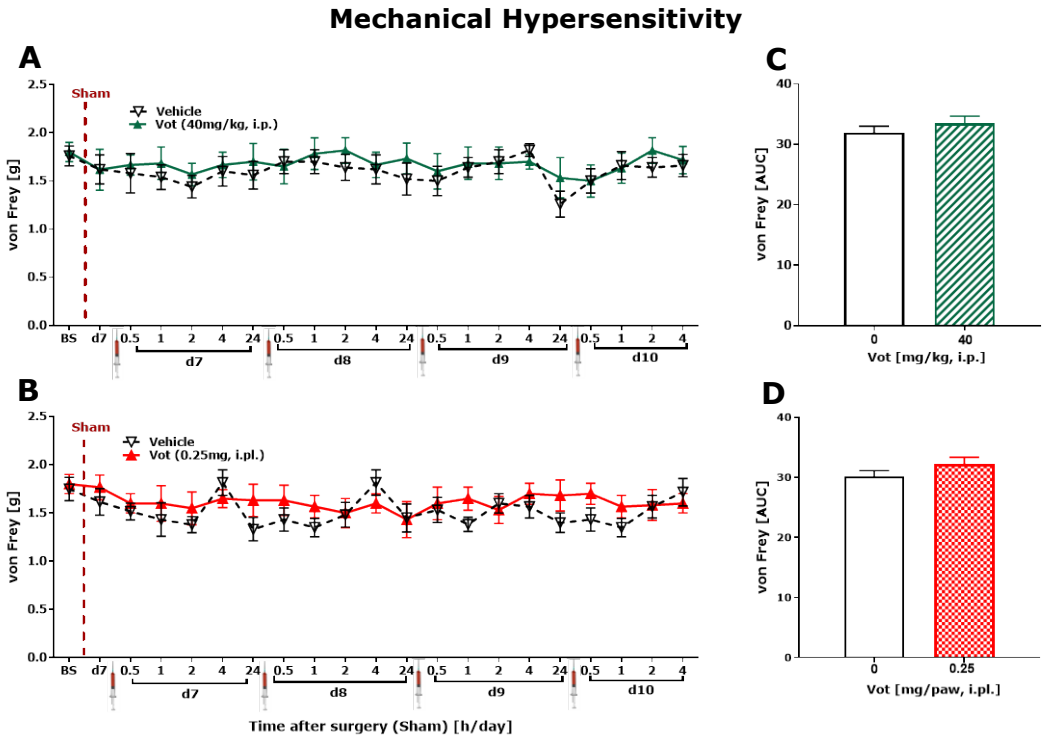


Figure 5.5 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of Votucalis did not attenuate overall mechanical hypersensitivity in sham mice. (A, B) Time-course effect of i.p. injection of Votucalis (Vot. 40 mg/kg, A) and i.pl. injection of Votucalis (Vot. 0.25 mg/paw, B) administered into the plantar surface of the injured paw, or vehicle (saline), on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5-24 hours after each of four consecutives i.p. (A) or i.pl. (B) injections. The syringe icon represents each of four i.p. (A) or i.pl. (B) injections of Votucalis. Data are presented as means ± S.E.M, n=6-8 in each group. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. Lack of significant changes *vs.* vehicle control animals; *p> 0.05 (two-tailed unpaired t-test).

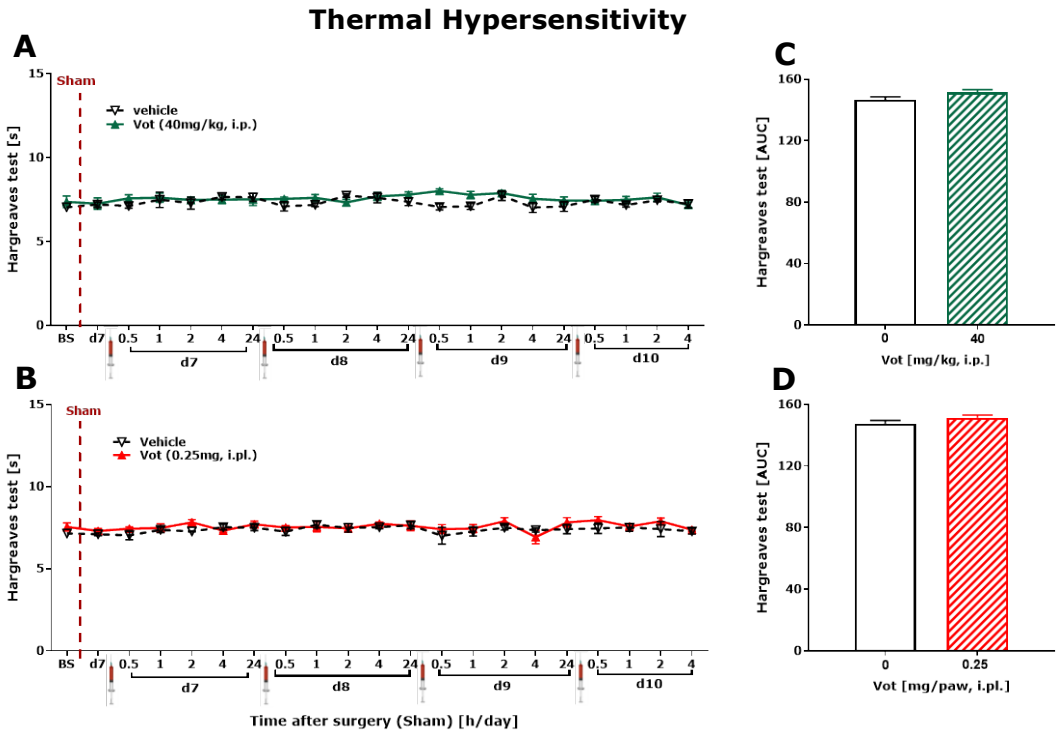


Figure 5.6 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of Votucalis did not attenuate overall thermal hypersensitivity in sham mice. (A, B) Time-course effect of i.p. injection of Votucalis (Vot, 40 mg/kg, A) and i.pl. injection of Votucalis (Vot, 0.25 mg/paw, B) administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the thermal withdrawal threshold measured with the Hargreaves test. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5-24 hours after each of four consecutives i.p. (A) or i.pl. (B) injections. The syringe icon represents each of four i.p. (A) or i.pl. (B) injections of Votucalis. Data are presented as means ± S.E.M, n=6-8 in each group. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. Lack of significant changes *vs.* vehicle control animals; (two-tailed unpaired t-test).

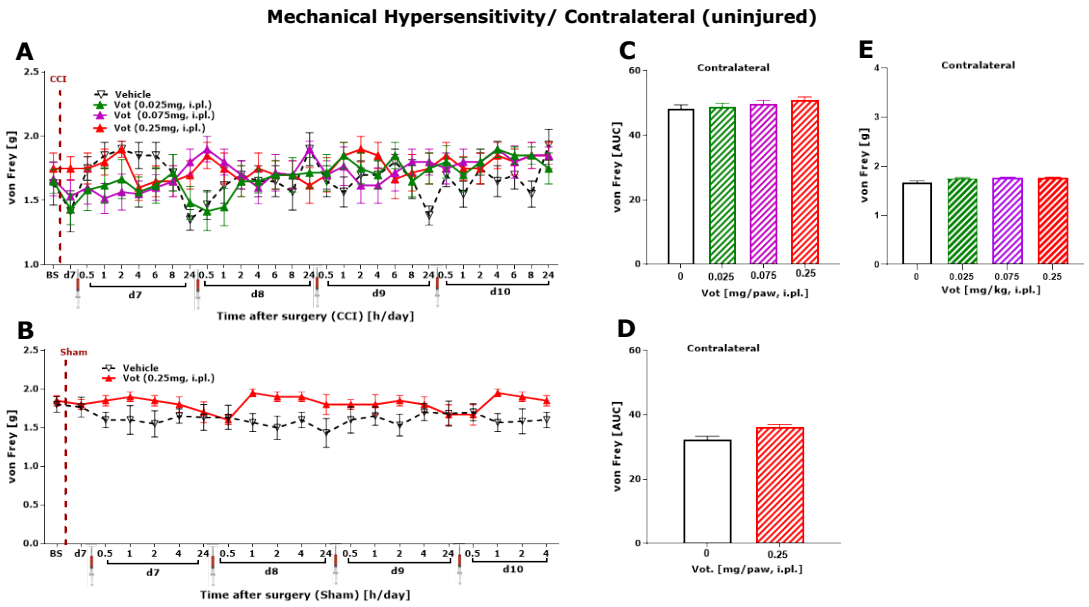


Figure 5.7 Chronic peripheral intraplantar (i.pl.) administration of Votucalis did not attenuate overall mechanical hypersensitivity in both chronic constriction injury (CCI) and sham mice at the contralateral hind paw. (A, B) Time-course effect of i.pl. injection of Votucalis (Votucalis , 0.025-0.25 mg/paw, A) and (Votucalis , 0.25 mg/paw, B) administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the mechanical withdrawal threshold measured with von Frey filaments n the contralateral hind paw (uninjured paw) . The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5-24 hours after each of four consecutives i.pl. (A) injections. The syringe icon represents each of four i.pl. injections of Votucalis. Data are presented as means ± S.E.M, n=6-8 in each group. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. (E) comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test. Lack of significant changes vs. vehicle control animals; for A,C: one-way ANOVA, followed by Bonferroni's comparison post-hoc test and for B,D: two-tailed unpaired t-test, E: repeated measure ANOVA followed by Bonferroni's test.

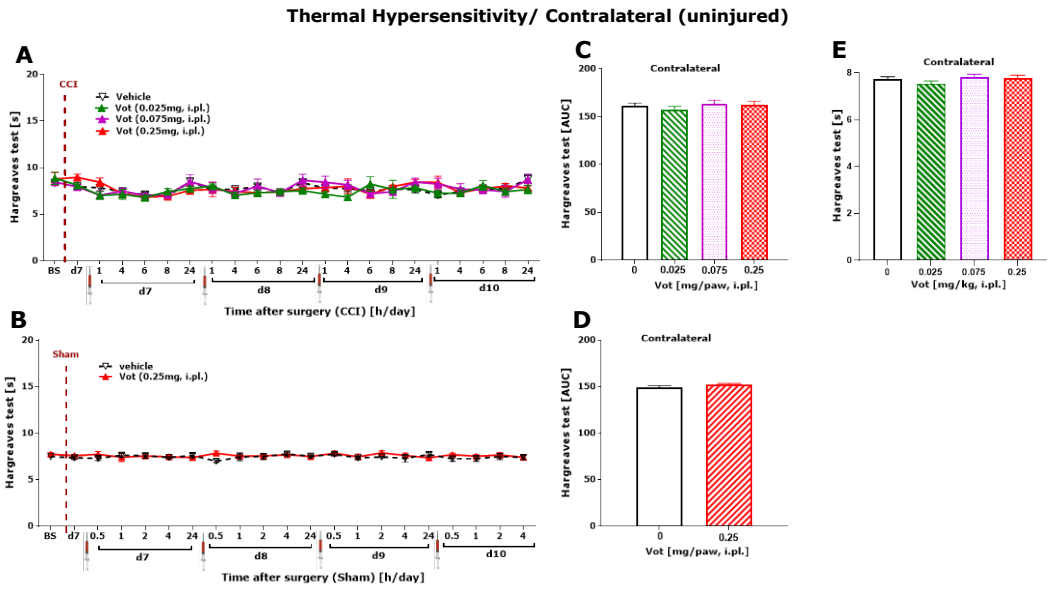


Figure 5.8 Chronic peripheral intraplantar (i.pl.) administration of Votucalis did not attenuate overall thermal hypersensitivity in both chronic constriction injury (CCI) and sham mice at the contralateral hind paw. (A, B) Time-course effect of i.pl. injection of Votucalis (Vot, 0.025-0.25 mg/paw, A) and (Vot, 0.25 mg/paw, B) administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the thermal withdrawal threshold measured with the Hargreaves test in the contralateral hind paw (uninjured paw) . The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5-24 hours after each of four consecutives i.pl. (A) injections. The syringe icon represents each of four i.pl. injections of Votucalis. Data are presented as means ± S.E.M, n=6-8 in each group. (C) The area under the curve (AUC) summarizing measurements in A. (D) The area under the curve (AUC) summarizing measurements in B. (E) comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test. Lack of significant changes vs. vehicle control animals; for A,C: one-way ANOVA, followed by Bonferroni's comparison post-hoc test and for B,D: two-tailed unpaired t-test, E, repeated measure ANOVA followed by Bonferroni's test.

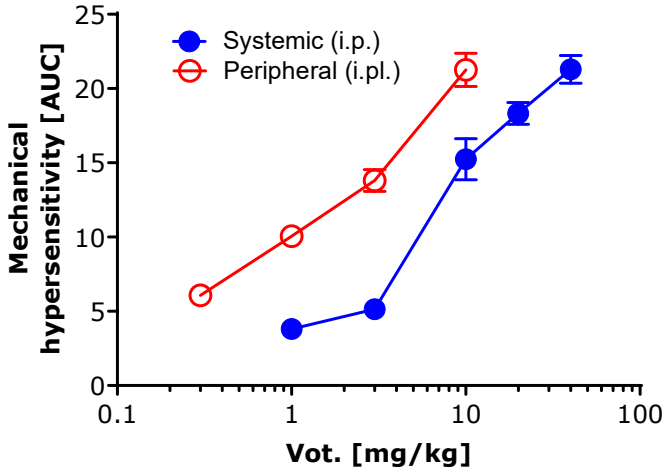


Figure 5.9 Peripheral (i.pl.) administration of Votucalis produced anti-nociceptive effects at a lower dose range compared to systemic (i.p.) administration. Dose-response effect of Votucalis (Vot, 0.3-40 mg/kg; intraperitoneal, i.p. (blue line) or intraplantar, i.pl. (red line) in the chronic constriction injury (CCI) model of neuropathic pain. Points represent the area under the curve (AUC) presented in Figure 5.1 and referring to changes in the mechanical withdrawal threshold measured with von Frey filaments. Data are presented as means ± S.E.M, n=6-14 in each group.

5.4 Discussion

The work presented in this Chapter explored the analgesic effects of Votucalis upon neuropathic pain that is related to mechanical and thermal hypersensitivity. Votucalis, centrally sparing and high-affinity recombinant histamine binding protein, after systemic and localised peripheral administrations almost completely blocked mechanical hypersensitivity in a pre-clinical model of neuropathic pain. These results highlight the importance of the histamine signaling pathway in regulating nociceptor sensitivity and may suggest this approach as a potential target for therapeutic intervention in neuropathic pain. Moreover, the findings clearly indicate that Votucalis after localised peripheral administration produced strong long-lasting anti-nociception at lower doses when compared to doses administered systemically. This observation provides the first evidence for targeting the peripherally released endogenous histamine by Votucalis suggesting a novel strategy for more efficacious, and potentially safer control of chronic neuropathic pain (Bennett and Xie, 1988; Obara et al., 2013)

In this study we found that CCI induced mechanical allodynia and thermal hyperalgesia responses in the injured hind-paw of the mice similarly to previous studies (Bennett and Xie, 1988; Obara et al., 2013; Zulazmi et al., 2015; Gopalsamy et al., 2019). Importantly, the mechanical withdrawal threshold and thermal withdrawal latency was observed on the first day after CCI started to decrease and existed for at least 10 days (end of our experiment) after the injury. According to the pathophysiology of neuropathic pain, it seems this type of pain is developed mainly as a result of the local inflammatory response that occurs in the affected area and subsequently release of wide range of mediators (Clatworthy et al., 1995). Inflammatory mediators released at the site of injury such as prostaglandins, substances P, cytokines and histamine bind to specific receptor located in the primary afferent nerve ending and induce local sensitisation of nerve fibres and generation an action potential that lead to increasing the firing rate along the nerve fibers (Zimmermann, 2001; Gopalsamy et al., 2019). All these changes lead to the development of hyperalgesia and allodynia which are two important characteristics of neuropathic pain (Zimmermann, 2001; Baron et al., 2010).

A number of studies have reported that peripheral histamine plays an important role in the physiological processes associated with neuropathic pain by stimulation and sensitization of histamine-sensitive fibres mainly C-fibres and Aδ-fibres (Parada et al., 2001; Farzin et al., 2002; Cannon et al., 2007). It has been reported that histamine released from neuronal and non-neuronal cells such as mast cells, basophils and eosinophils at the site of nerve injury leads to the development and maintenance of mechanical and thermal hyperalgesia (Khalilzadeh et al., 2018). After histamine is released, it works by bind to one of the four distinct GPCRs known as the histamine $H_{1-4}R$. H_1R ,H_3R and H_4R are believed to be involved in nociceptive transmission (Hough and Rice, 2011; Popiolek-Barczyk et al., 2018) while the involvement of H_2R in the pain sensation, particularly, neuropathic pain is somewhat contradictory (Farzin et al., 2002; Khalilzadeh et al., 2018).

The interaction between histamine and the receptors lead to the action of the receptors and activation of the downstream signalling pathway leading to an increase in Ca^{2+} influx and causing activation of histamine-sensitive C-fibers (Kim et al., 2004; Khalilzadeh et al., 2018). In addition, it was shown that histamine contributes to neuropathic pain mechanism by increasing voltage gated Na^+ channels, in particular Nav1.8 and Nav1.9 expression in primary afferent neurons and L4/L5 DRG neurons (Yue et al., 2014; Bennett et al., 2019). Moreover, it has been demonstrated that histamine released by activated mast cells stimulates the recruitment of leukocytes into the sites of peripheral nerve injury (Gaboury et al., 1995, Yong et al., 1997, Yamaki et al., 1998). Indeed, it was shown that histamine may cause the release of different type of inflammatory mediators such as leukotrienes, cytokines, and chemokines that causes the sensitization of transient receptor potential vanilloid type 1 (TRPV1) (Jemima et al., 2014; Gao et al., 2016 ; Jardín et al., 2017; Thangam et al., 2018). In addition, histamine can act on non-histaminergic pathway such as activation of extracellular signal-regulated kinase (ERK) and N-methyl-d-aspartate receptors (NMDAR) which are responsible for the transduction of nociceptive signals (Peng et al., 2009; Aiyer et al., 2018). All these mediators and channels can be activated by histamine playing a significant role in the inflammatory response which may result in the development of neuropathic pain (Schomberg et al., 2012; Zhang et al., 2019; Thangam et al., 2018).

The concept of peripheral targeting approaches is consistent with current theories of mechanism-based pain treatment. As a result, they have the potential to improve the quality of pain management and the satisfaction of patients with their treatment (Müller-Schwefe et al., 2017; Kocot-Kępska et al., 2021). Several clinical studies show that targeting peripheral mechanism using topical treatments or subcutaneous injection of lidocaine patch, capsaicin patch, and botulinum toxin A have comparable analgesic efficacy in patients with neuropathic pain but have fewer drug–drug interactions and systemic side effects when compared to systemic administration (Kocot-Kępska et al., 2021). From that point, targeting localized peripheral histamine at the site of injury and its signaling pathways may have beneficial effects on the mechanism of chronic neuropathic pain, and may also provide promising therapeutic potential for novel drugs in this condition.

Votucalis, is a histamine binding protein that act as a histamine scavenger, binding to the histamine molecule and leading to neutralization of endogenous histamine and inhibition of the recruitment of the inflammatory mediators at the site of disease or injury (Weston-Davies et al., 2005). In this study, it is probable that Votucalis neutralised endogenously released histamine causing a potent and dose-dependent anti-nociceptive effect. Interestingly, while mechanical hypersensitivity was blocked by peripherally administered Votucalis, heat hypersensitivity remained unaffected by this treatment. This specific anti-nociceptive effect may suggest involvement of H_3R and that Votucalis prevents histamine binding to H_3R. Since both pharmacological and genetic alterations of H_3R activity have confirmed its importance and specificity for mechanical hypersensitivity (more detail will be discussed in next Chapter) (Cannon et al., 2003; Wei et al., 2016). In addition, anatomical studies have confirmed localisation of H_3R receptors on Aδ-fibres that conduct tactile sensation (Cannon et al., 2007; Lawson, 2002) as well as the ability of H_3R antagonists to block secondary mechanical hypersensitivity (Medhurst et al., 2007). Based on that fact, this work may suggest that Votucalis, by scavenging endogenous histamine, reduced the sensitivity of H_3R-positive A-fibres resulting in a diminished input to the dorsal horn, supporting the potential role

for H_3R receptors in the modulation of central sensitization. In contrast, heat hypersensitivity is regarded as a sign of the peripheral sensitization of C-fibres, which do not express H_3R receptors (Cannon et al., 2007).

Taking all the results together, these results provide the first evidence for the analgesic effects of Votucalis upon neuropathic pain related mechanical hypersensitivity and thus emphasize the importance of histamine in the modulation of peripheral neuropathies. Direct targeting of histamine itself by sequestering the ligand with Votucalis may represent a new tool to the control histamine-dependent pain mechanism, particularly after its localised administration. This approach of ligand capture may be superior to the antagonism of single receptors and may support our hypothesis of the role of peripheral histamine in chronic pain. Given that nerve injury-induced mechanical hypersensitivity involves A-δ fibres that were shown to express H_3R, our result may suggest that Votucalis analgesic effects may prevent binding histamine to HR particularly after local peripheral administration. Complicated peripheral mechanisms following nerve injury are caused for the development and maintenance of pain and sensory abnormalities in patients with neuropathic pain (Raja et al., 2020). Therefore, the use of local peripheral analgesics that act locally at the peripheral level is justified in accordance with current concepts in pain medicine, which emphasize the importance of an individualized and mechanism-based approach in pain management (Müller-Schwefe et al., 2017; Kocot-Kępska et al., 2021). It is expected that topically applied analgesics will only target the underlying molecular/cellular mechanisms in the periphery, with no consideration given to systemic mechanisms.

The results in this Chapter encouraged us to further investigate the importance of peripheral H_3R in the modulation of chronic pain. We therefore examined the effects produced by selective and peripherally acting/centrally sparing H_3R ligand PF-8680872 in a model of peripheral neuropathic pain and these data are presented in detail in the next Chapter.

Effect of systemic and local peripheral administration of a novel CNS-sparing H_3R antagonist PF-0868087 on neuropathic pain

6.1 Introduction

In the previous Chapter, the significance of peripherally released histamine in the regulation of neuropathic pain has been demonstrated based on animal studies using the histamine binding protein Votucalis, targeting histamine itself *via* scavenging its endogenous release. The data presented in the previous Chapter, may suggest involvement of peripheral H_3R in the regulation of mechanical hypersensitivity in neuropathic pain. Thus, this Chapter is focussed on exploration the role of peripheral H_3R in the modulation of neuropathic pain by using a selective CNS-sparing H_3R antagonist in mice.

As described in the first chapter, neuropathic pain is a debilitating and refractory disease characterized by the occurrence of hyperalgesia and allodynia. This type of chronic pain is produced by injury to either peripheral or central somatosensory nervous system. The symptoms of neuropathic pain vary significantly depending on the type of nerves injured. Additionally, the signs and symptoms are not constant but change over time (Ueda, 2006). When a peripheral nerve is injured, functional and biochemical changes occur not only at the site of the injury, but also in other parts of the affected nerves and, eventually, in higher-order neurons in the CNS (Bridges et al., 2001, Lai et al., 2001; Ueda, 2006). Neuropathic pain is associated with reduced functional status and lower treatment compliance which leads to poor quality of life, negatively affecting the cognitive, emotional and behavioural aspect of patient's life and their families (Dueñas et al., 2016; Torta et al., 2017). However, till date the pathophysiological mechanisms underlying neuropathic pain are not fully understood. Various changes in pathophysiological and biochemical systems can lead to morphological and functional alterations in both PNS and CNS, involving a rise in excitatory neurotransmitters and neuropeptides, like 5-HT, glutamate and histamine, leading to the development of hyperexcitability (Singh et al., 2011; Obara et al., 2020).

Despite an increase in basic and clinical knowledge of the aetiology and diagnosis of neuropathic pain, the current available therapy for this type of chronic pain remains insufficient. To date, no medication has proved tolerability and long-term efficacy for neuropathic pain and with existing medications, approximately less than 50% of patients achieved control of pain (Ciaramitaro et al., 2019; Sałat, 2020). Most of these current medications, in particular, systemic treatment options come with potential side effects and drug interactions especially with prolonged usage and polypharmacy (Chong and Bajwa, 2003; Haanpää *et al.*, 2010; Bates *et al.*, 2019; Dickenson and Patel, 2020). In additions, some pain management clinics used trial and error approach for the management of neuropathic pain but this approach is not based on any scientific approval or policy so it may be beneficial for some patients, but not for others (Watson and Watt-Watson, 1999; Finnerup and Attal, 2016). Therefore, there is a demand for novel therapeutic agents that work at a new target site with a novel mechanism.

Recently, researchers have discovered a link between chronic neuropathic pain and the histamine system. Histamine system is being targeted for several other therapeutic interventions such as sleep disorders, schizophrenia, depression, Alzheimer's disease, Parkinson's disease, epilepsy and eating disorders (Haas et al., 2008; Shim and Oh, 2008). It is also being targeted for other peripheral disorders such as acute asthma, allergic conjunctivitis, itch, and chronic neuropathic pain (Wang et al., 2016; Branco et al., 2018; Tatarkiewicz et al., 2019). However, outcomes stating the effects of the histamine system in chronic neuropathic pain are contradictory. This inconsistency in the effect of histamine in chronic neuropathic pain might be related to many reasons like affinity of the receptors, distribution of HR and their pharmacological properties, or maybe due to the access of the drugs targeting the histamine system to both the CNS and PNS. It is important to note that histamine interacts with its receptors in a variety of ways with a different affinity (Tatarkiewicz et al., 2019). H_3R and H_4R are the most sensitive to the histamine with very low concentration, whereas the activation of H_1R and H_2R needs much higher concentration levels of histamine (Tiligada et al., 2009). In some diseases for which traditional antihistamines, H_1R antagonists were ineffective, novel histamine receptor ligands acting on H_3R may be effective (Cataldi et al., 2014; Tatarkiewicz et al., 2019).

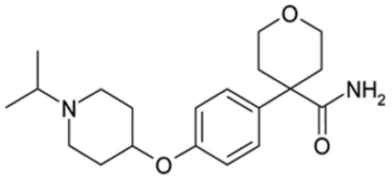
In general, excitatory HR signaling in nociceptive pathways is associated with increased pain symptoms (Gangadharan and Kuner, 2013; Mobarakeh et al., 2000), whereas inhibitory HR signaling in nociceptive pathways is associated with pain relief (Gangadharan and Kuner, 2013; Mobarakeh et al., 2000; Chazot and Care, 2005; Esbenshade et al., 2008; Bhowmik et al., 2012). In contrast to this general function, the role of H_3R in the perception of pain continues to be debated. H_3R is found in both the CNS and PNS, with the majority of its expression occurring in ascending and descending nociceptive pathways (Cannon et al., 2007; Heron et al., 2001; Obara et al., 2020). Depending on the type of model used, the type of nociceptive stimulation used in these studies, and the route of administration of the tested drug (systemic vs. peripheral), stimulation of H_3R has been shown to either cause allodynia or attenuate hyperalgesia (Huang et al., 2007; Popiolek-Barczyk et al., 2018; Smith et al., 2007).

Several academic groups and pharmaceutical industry began to focus their attention on H_3R since the time of cloning of H_3R in 1999, to discover and develop ligands for H_3R to cure several diseases including neuropathic pain (Lovenberg et al., 1999; Obara et al., 2020). Interestingly, it also functions as hetero-receptors and regulates release of other neurotransmitters such as dopamine, serotonin, norepinephrine, glutamate, noradrenalin, and γ-aminobutyric acid (GABA) (Blandina et al., 2010, Cowart et al., 2012, Haas et al., 2008). Thus, stimulation of H_3R leads to inhibition of histamine synthesis and release from synaptic vesicles of the neurons *via* negative feedback mechanism (Arrang *et al.*, 1983; Morisset *et al.*, 2000; Wang *et al.*, 2020). Moreover, accumulating evidence suggests that the H_3Rs are expressed in various non-brain tissues, such as stomach, intestines, adipose tissue, heart, lung, dorsal root ganglia, superior cervical ganglia, skin and on specific types of primary sensory neurons like Aβ fibers and Aδ fibers terminating on deep dermal blood vessels (Cannon et al., 2007; Obara et al., 2020; Heron et al., 2001; Hough and Rice, 2011).

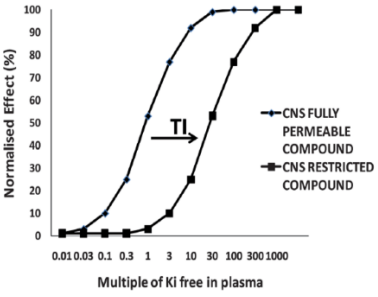
H_3R ligand ZPL-868087 (also known as ZPL-389) was recently identified as a selective and peripherally acting/CNS-sparing ligand with no significant effect at the other receptor subtypes (Lunn et al., 2012). It should be noted that the UK Pfizer developed ZPL-868087 presently owned by Novartis and is called 'PF-0868087'. The chemical structure of the drug is shown in Figure 6.1. This drug is unique in the way that it allows

limited penetration to the CNS thus minimising the risk of potentially harmful CNS related adverse effects (Lunn et al., 2012).

A



B



C

Compound	PF-0868087
$logD_{pH7.4}$	0.0
Topological polar surface area (Å^2)	65
Solubility (mg ml^{-1})	>1
RRCK Papp ($\times 10^{-6}$ cm s^{-1})	2
MDCK-MDR1 efflux ratio	3.3
RRCK-BCRP efflux ratio	5.6
[CSF]/[Plasma] (free) ratio rat after 4 h *iv* infusion	0.06
[Brain](free)/[plasma](free) ratio rat after 6 h *iv* infusion	0.1
[Brain](free)/[plasma](free) ratio rat after 8 day oral dosing[c]	0.07
[Brain](free)/[plasma](free) ratio dog after 8 day oral dosing[d]	0.1
EEG PKPD rat study TI relative to CNS penetrant compound Z	16
Oral bioavailability, in rat. F (%)	42

D

Dose	Route	Total C_{max} (ng mL^{-1})	Free C_{max} (nM)	T_{max} (h)	AUC (ng hr mL^{-1})	CL (mL min^{-1} kg^{-1})	V_{ss} (L kg^{-1})	Effective $T_{1/2}$ (h)	Absorption (%)	F (%)
1 mg kg^{-1} Rat (n = 3)	*iv* bolus	477	856	—	776	27	4.1	1.9		—
2 mg kg^{-1} Rat (n = 3)	*po*	89	158	0.75	649	—	—	—	57	42
0.2 mg kg^{-1} Dog (n = 2)	*iv* infusion	129	320	—	548	6.1	2.3	4.5		
0.2 mg kg^{-1} Dog (n = 2)	*po*	142	352	0.90	727	—	—	—	Complete	133

Figure 6.1 PF-0868087, structure and pharmacokinetic properties. (A) PF-0868087 was originally developed by Pfizer and Ziarco, and currently is owned by Novartis. It is selective for H_3R and functions as a selective antagonist (reversible inverse agonist) at H_3R. **(B)** Representation of normalised effect versus plasma exposure for H_3R antagonists for a fully CNS permeable reference compound and a compound with restricted exposure to the CNS. This confirms the uniqueness of this drug that lies in its chemical and morphological structure allowing for restricted penetration to CNS to minimise CNS-related adverse effects. **(C)** PF-0868087 is a compound with low permeability, high solubility, and is a substrate for the blood-brain-barrier efflux transporters MDR-1 and BCRP. It displays a greater than 10-fold CNS-sparing profile in pre-clinical species shown by ([brain](free)/[plasma](free)) ratio in rat and dog after 8-day oral dosing. **(D)** Summary of blood pharmacokinetics of PF-0868087 in a rat pre-clinical species. In summary, the pre-clinical profile of PF-0868087 showed its high tolerability and suitability for oral dosing thus further supporting the potential for PF-0868087 as a clinical strategy for improving neuropathic pain control. (adapted from Lunn et al., 2012).

With the application of PF-0868087 in clinical trial on allergic rhinitis, and with its H_3R selectivity and high solubility, favourite pharmacological and chemical profile (Lunn et al., 2012) and including the previous data collected, it indicates that there is no relevant drug–drug interaction with PF-0868087 and the drug is generally well tolerated. In addition, pre-clinical study showed that no toxicological signs were observed in rats after

a daily administration of PF-0868087 for 7 days (Lunn et al., 2012). PF-0868087 was well tolerated up to no observable effect level (NOEL) at a dosage of 80 mg/kg/day for 7 days (Lunn et al., 2012). All these major advantages represent this drug as perfect and ideal candidate to be a new tool for validation of peripheral H_3R as a potential target for therapeutic involvement in chronic pain, particularly in peripheral neuropathic pain.

The study in this Chapter focuses on the role of H_3Rs, particularly peripheral H_3Rs, in neuropathic pain. This type of receptor represents a novel and tractable target for the treatment of neuropathic pain originating within the spinal somatosensory system. Specifically, we postulate that H_3R antagonism induced by peripherally acting PF-0868087 leads to blocking mechanical hypersensitivity associated with neuropathic pain . Importantly, this effect can potentially be achieved by using the clinically utilised drug, thus validating a novel and clinically viable avenue for direct application in humans.

6.2 Materials and Methods:

6.2.1 Subjects

Experiments in this chapter were carried out using adult male C57BL/6J mice (8 weeks of age; 20–25 g; Charles River Laboratories, Harlow, UK) as previously described in Chapter two (Section 2.2). Animals underwent surgery for a model of experimental mononeuropathy, a unilateral chronic constriction injury to the sciatic nerve (CCI; Section 2.5.1) and were behaviourally assessed (Section 2.6) prior to surgery (to obtain baseline values) and post-surgery (from day 6) until (day 10 post surgery) from sham and CCI. All animals were behaviourally assessed (Section 2.6) prior to injection (to obtain baseline values) and post injection. All animals were tested for signs of mechanical allodynia using calibrated von Frey filaments (Section 2.6.2) and for signs of thermal hyperalgesia using the Hargreaves' thermal apparatus (Section 2.6.3). Data was collected from both the ipsilateral and contralateral paws. Each animal first underwent von Frey testing followed by the Hargreaves test. A schematic diagram of this experiment is shown in Figure 6.2.

6.2.2 Preparation and administration of drugs

PF-0868087 was provided by Ziarco Pharma (UK) as a pure crystalline powder and stored in adequate conditions (protected from humidity and light) in the refrigerator

between 2 and 4°C. For all administrations, PF-0868087 was prepared immediately before injections and dissolved in a vehicle (sterile 0.1 M citrate buffer solution) at required concentrations as described below.

Systemic intraperitoneal (i.p) administration. Mice were weighed and randomized to receive either PF-0868087 or vehicle (citrate buffer); they were injected i.p. with PF-0868087 at 1, 3, and 10 mg per kg body weight or vehicle (0.1M citrate buffer) solution without PF-0868087 as a control group . PF-0868087/vehicle was administered once every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. The animals received a total of 4 i.p. injections of PF-0868087/vehicle.

Peripheral intraplantar (i.pl.) administration. Mice were randomised to receive either PF-0868087 or vehicle (0.1 M citrate buffer); they were injected i.pl. with PF-0868087 at 0.003, 0.03 and 0.3 mg/paw or equivalent citrate buffer solution without PF-0868087 as a control group. Injections were given over 1 min in a volume of 50 µL without anaesthesia into the plantar surface of the animal hind paw ipsilateral the sciatic nerve injury. PF-0868087/vehicle was administered once every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. The animals received a total of 4 i.pl. injections of PF-0868087/vehicle.

Peripheral subcutaneous (s.c.) administration. Mice were weighed and randomized to receive either PF-0868087 or vehicle (0.1 M citrate buffer); they were injected s.c. into the nape of the mouse neck with PF-0868087 at 0.3 mg per kg body weight or vehicle (0.1 M citrate buffer) solution without PF-0868087 as a control group. PF-0868087/vehicle was administered once every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. The animals received a total of 4 s.c. injections of PF-0868087/vehicle.

All other chemicals including, sodium citrate dihydrate, hydrochloric acid, citric acid, and sodium hydroxide, were obtained from Sigma-Aldrich Chemical Co, UK. Drugs were injected in a volume of 100 µl for i.p. administration and of 50µl for s.c. and i.pl. administrations.

5.2.3 Neuropathic pain model

5.2.3.1 Induction and assessment of neuropathic pain

The method for the induction, and assessment of neuropathic pain is described in detail in Chapter 2.

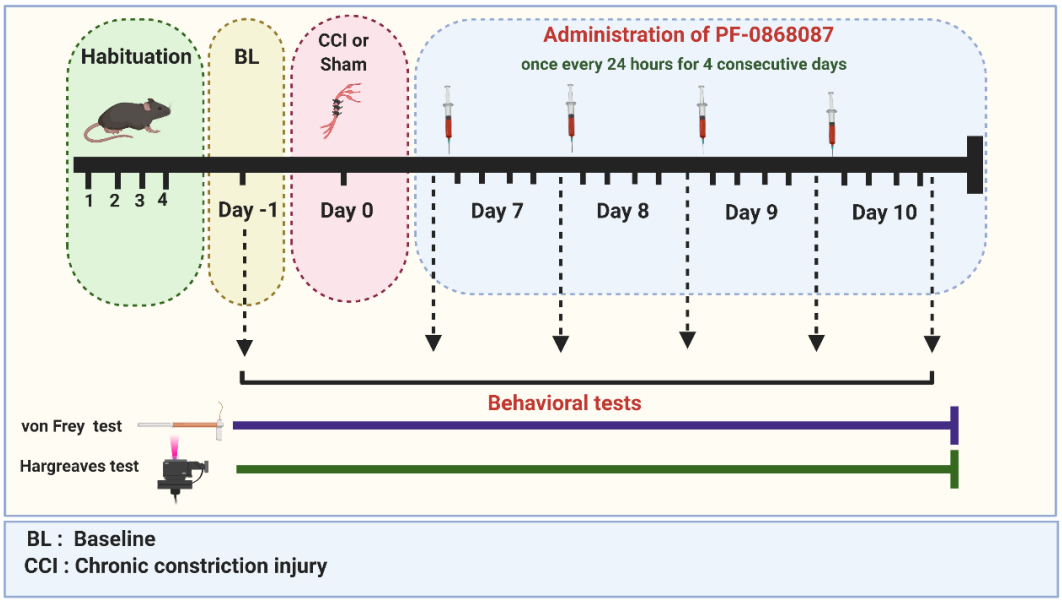


Figure 6.2. A schematic diagram summarising timeline of the experiment. Representation of the experimental schedule which shows neuropathic pain (CCI) induction (day 0), PF-0868087 treatments (days 7, 8, 9 and 10 post CCI) and behavioural assessment for mechanical and thermal stimulation thresholds that were taken one day before CCI as baseline and then repeated consecutively after administration of PF-0868087 30 min, 1 and 24 h for a period of 4 days.

6.2.4 Design of the experiments

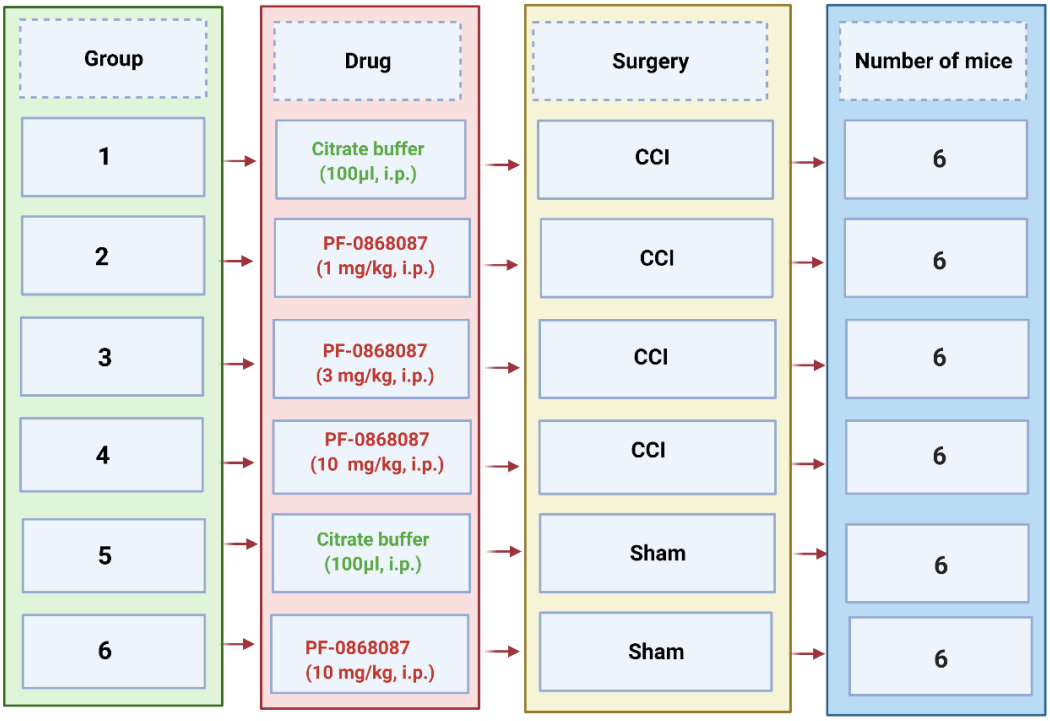
6.2.4.1 To prove the anti-nociceptive efficacy of PF-0868087 after systemic and local peripheral administration three experiment were conducted

Experiment 1: Anti-nociceptive efficacy of systemic administration of PF-0868087

In this experiment, CCI mice were divided into 6 different groups as indicated in Table 6.1. Baseline (basal pain threshold) for mechanical and thermal hypersensitivity thresholds were taken one day before CCI and sham surgery on (day -1). Both mechanical and thermal hypersensitivity were measured at the lateral plantar surface of the hind paw before nerve injury and then the behavioural tests were repeated on

day 7 post surgery (CCI and sham) and continued for 3 consecutive days on day 8, 9 and 10. Both tests were performed prior the administration of PF-0868087 i.p. and were repeated at 30 min, and 1 and 24 hours after each of PF-0868087 administrations across four test days. Each animal first underwent von Frey testing followed by the Hargreaves test. PF-0868087/vehicle was injected once daily at the same time every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. Sham control mice were divided into 2 groups (group 6 and 7) as indicated in Table 6.1. The behavioural tests and treatment schedule were done as described in CCI group.

Table 6.1 Experimental groups in experiment 1.

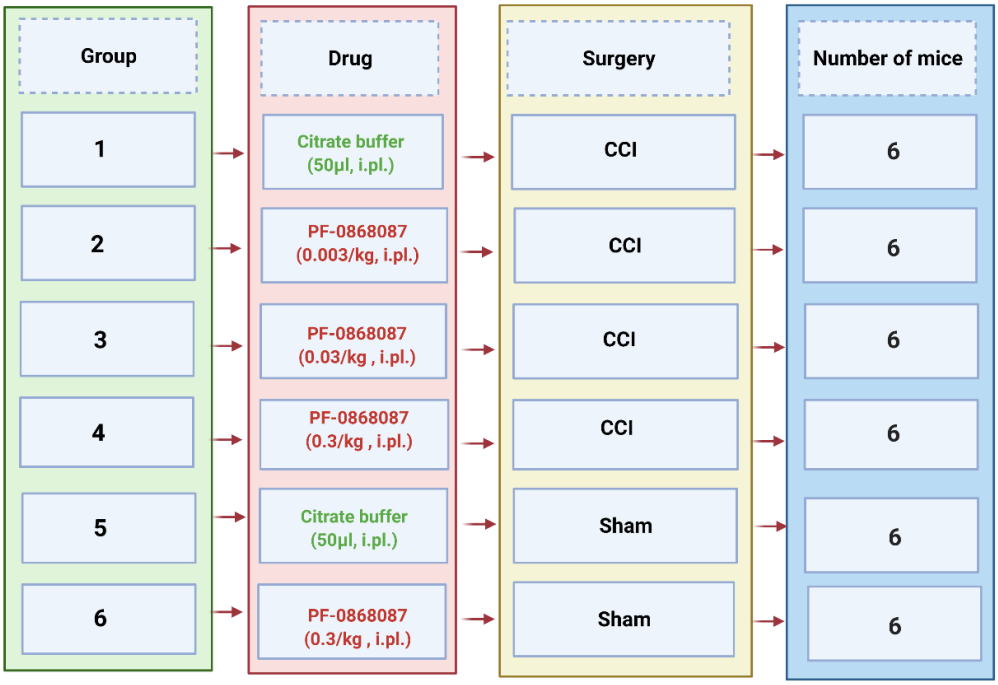


CCI: Chronic Constriction Injury, **i.p.:** intraperitoneal administration
CCI and sham mice were randomly assigned into 6 groups (n=6), received different doses of PF-0868087/vehicle (citrate buffer) control as following **(Group 1)** CCI mice received systemic i.p. injection of vehicle (citrate buffer) in a volume of 100 µL; **(Groups 2-4)** CCI mice received i.p. injection of different doses of PF-0868087 (1,3,and 10mg/kg); **(Group 5)** Sham mice received i.p. injection of vehicle (citrate buffer) in a volume of 100 µL; **(Group 6)** Sham mice received systemic i.p. injection of PF-0868087 (10mg/kg).

Experiment 2: Anti-nociceptive efficacy of Peripheral administration of PF-0868087

In this experiment CCI mice were divided into 6 different groups as indicated in Table 6.2. Baseline (basal pain threshold) for mechanical and thermal hypersensitivity thresholds were taken one day before CCI and sham surgery on (day -1). After that, the behavioural tests were repeated on day 7 post surgery (CCI and sham) and continued for 3 consecutive days on day 8, 9 and 10. Both tests were performed before localised peripheral administration of PF-0868087 (i.pl.) and were repeated at 30 min, 30 min, and 1 and 24 hours after each PF-0868087 administrations across four test days. Each animal first underwent von Frey testing followed by the Hargreaves test. PF-0868087/vehicle was injected once daily at the same time every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. Sham control mice were divided into 2 groups (group 6 and 7) as indicated in Table 6.2. The behavioral tests and treatment schedule were done as described in CCI group.

Table 6.2 Experimental groups in experiment 2.



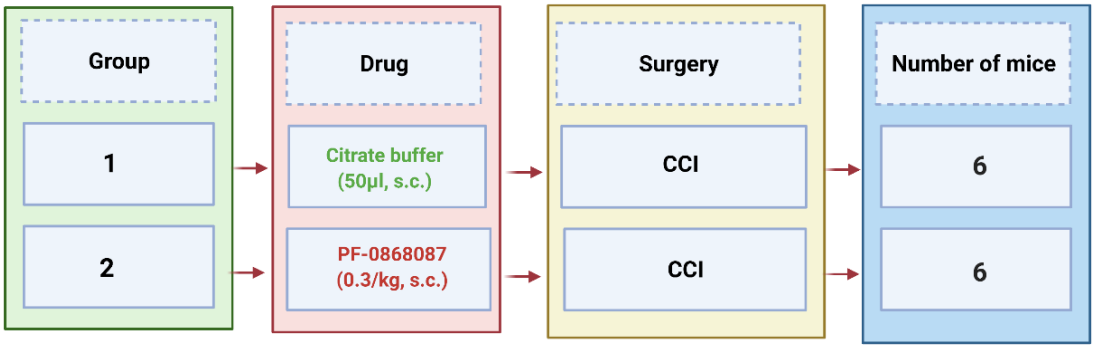
CCI: Chronic Constriction Injury, **i.pl.:** intraplanter administration
CCI and sham mice were randomly assigned into 6 groups (n=6), received different doses of PF-0868087/vehicle (citrate buffer) control as following **(Group 1)** CCI mice received i.pl. injection of vehicle (Citrate buffer) in a volume of 50 µL; **(Groups 2-4)** CCI mice received local i.pl. injection of

different doses of PF-0868087 (0.003, 0.03mg,and 0.3mg/kg); **(Group 5)** Sham mice received i.pl. injection of vehicle in a volume of 50 µL; **(Group 6)** Sham mice received local i.pl. injection of PF-0868087 (0.3mg/kg).

Experiment 3: Anti-nociceptive efficacy of peripheral administration of PF-0868087

This experiment aimed to confirm that the anti-nociceptive efficacy of peripheral administration (i.pl.) of PF-0868087 (Experiment 2) was due to blocking the histamine receptors at the site of the injection. Thus, in this experiment CCI mice were divided into 2 different groups as indicated in Table 6.3 and the effect observed after s.c. administration of PF-0868087 to the area distal from site of injury were compared to the effect produced by the same dug and dose after i.pl. administration. The behavioral tests and treatment schedule were performed as previously described.

Table 6.3 Experimental groups in experiment 3.

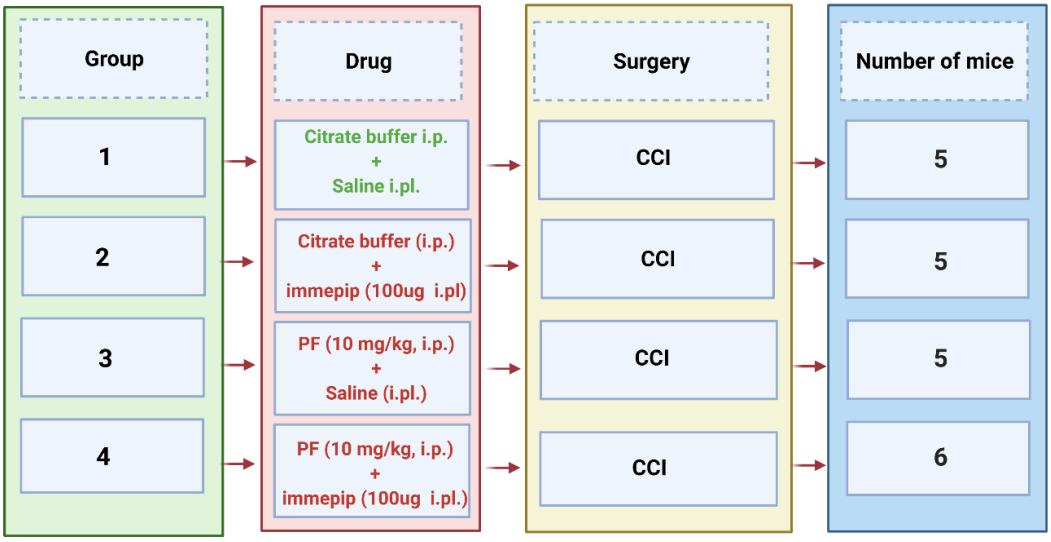


CCI: Chronic Constriction Injury, **s.c.:** subcutaneous administration
CCI mice were randomly assigned into 2 groups (n=6), received selected doses of PF-0868087/vehicle (citrate buffer) control as following **(Group 1)** CCI mice received local s.c. injection of vehicle (Citrate buffer) in a volume of 50 µL; **(Group 2)** CCI mice received local s.c. injection of PF-0868087 (0.3mg /kg).

6.2.4.2 To prove the role of peripheral H_3R in mechanical hypersensitivity

In this experiment, CCI mice were divided into 4 different groups as indicated in Table 5.4. Baseline (basal pain threshold) for mechanical and thermal hypersensitivity thresholds were taken one day before CCI and sham surgery (day -1). Both mechanical and thermal hypersensitivity at the lateral plantar surface of the hind paw were assessed before the nerve injury (as basal pain threshold) and then the behavioral tests were repeated on day 7 post surgery (CCI and sham) and continued for 3 consecutive days on day 8, 9 and 10. Both tests were performed prior to the administration of PF-0868087 (i.p.) alone or 30 minutes before injection of the H_3R agonist, immepip 100µg (i.pl.) and were repeated at 1 and 24 hours after each treatment on the four test days. Each animal first underwent von Frey testing followed by the Hargreaves test. PF-0868087/vehicle was injected once daily at the same time every 24 hours in days 7, 8, 9 and 10 post induction of neuropathic pain.

Table 6.4 Experimental groups in experiment 4.



CCI: Chronic Constriction Injury, **i.pl.:** intraplanter administration, **i.p.:** intraperitoneal administration CCI mice were randomly assigned into 4 groups (n=5-6), received selected doses of PF-0868087/vehicle control with or without immepip (100µg) as following **(Group 1)** CCI mice received systemic i.p injection of citrate buffer solution in a volume of 100 µL, 30 minutes before i.pl. administration of saline as control; **(Group 2)** CCI mice received systemic i.p. injection of citrate buffer solution in a volume of 100 µL, 30 minutes before i.pl. injection of immepip (100µg) in a volume of 50 µL; **(Group 3)** CCI mice received i.p. injection of PF-0868087 (10mg/kg) in a volume of 100 µL, 30 minutes before i.pl. administration of saline. **(Group 4)** CCI mice received systemic i.p injection of PF-086808 (10mg/kg) in a volume of 100 µL, 30 minutes before i.pl. injection of immepip (100µg) in a volume of 50 µL.

6.3 Statistical analysis

All the data analysis and statistical comparisons were made using GraphPad PrismTM, (version 9.1.2) for Windows/OS (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The results of behavioural changes are presented in the graphs as mean ± SEM. Each group included 6 mice. Statistical analysis was performed by one- or two-way analyses of variance (ANOVA) with Bonferroni's multiple comparison post-hoc tests or by unpaired Student's t-test when two groups were compared. Repeated measures ANOVA used where drug effects were quantified repeatedly over a period of time. A value of $p < 0.05$ *vs* respective control group was considered to be statistically significant.

6.4 Results

6.4.1 CCI induced neuropathic pain in mice

CCI procedure led to the development of both mechanical allodynia and thermal hyperalgesia on the mid-plantar paw area supplied by the sciatic nerve as demonstrated in Figure 6.1. Neuropathic sensitisation of behavioural reflexes, characteristic of the CCI model, were assessed at 6-7 days after surgery. As illustrated in Figure 6.3 (A and E), the development of behavioural signs of neuropathic pain on the ipsilateral side after the nerve injury were observed as mechanical allodynia ($t_{(70)}$= 31.66, P<0.0001, n=6, A), and thermal hyperalgesia ($t_{(70)}$=13.06, P<0.0001, n=6, E). Indeed, no change in mechanical or thermal hypersensitivity from baseline in contralateral sides of CCI mice were observed (Figure 6.2 B and F). Moreover, mice underwent sham surgery (control), showed no change from baseline threshold in both ipsi- (Figure 6.2 C and D) and contralateral sides (Figure 6.2 G and H). Apart from these changes, post-operative animals appeared healthy, had no signs of weight loss and were handled without any distress being evident. Also, during this study none of the animals developed any signs of autotomy.

6.4.2 Systemic and peripheral administrations of PF-0868087 significantly attenuated mechanical hypersensitivity in neuropathic mice

The analgesic effectiveness of systemic (i.p) administration of PF-0868087 was assessed by measuring the paw withdrawal threshold in response to mechanical stimuli using von Frey filaments in mice with neuropathic pain (CCI model) 7 days after surgery. After systemic administration the mechanical hypersensitivity in CCI mice was significantly reduced with the two highest doses of PF-0868087 (3 and 10 mg/kg), (6.8±0.9 and 7.1±1, respectively) compared to vehicle controls alone (2.9±0.6) (Figure 6.4 A, C; effect of the drug: $F_{(3,20)}$= 10.16, P<0.001, n=6). The highest reduction in mechanical hypersensitivity was observed 0.5-1 h after the first injection of PF-0868087 (3 and 10 mg/kg) when compared with vehicle controls. However, 24 hours after the administration of PF-0868087 the effect was not significant. A similar pattern of analgesic effect was observed after each of four consecutives daily systemic administration of PF-0868087 (3 and 10mg/kg; Figure 6.4, A, D n=6). No significant difference in mechanical hypersensitivity was observed with the lowest dose of PF-0868087 (1 mg/kg) (2.3±0.4)

when compared to control group (2.9±0.6) (Figure 6.4. A and C; P>0.05, n=6).

After peripheral (i.pl.) administration of PF-0868087 the mechanical hypersensitivity in CCI mice was significantly reduced with the two highest dose of PF-0868087 0.03 and 0.3 mg/paw, (11.5±1.2 and 12.7±1.2, respectively) when compared to vehicle controls alone(2.9±0.3) (Figure 6.4 B and D, P<0.0001, n=6). The greatest reduction in mechanical hypersensitivity was observed 0.5-1 hours after the first injection of PF-0868087 (0.03 and 0.3 mg/paw) when compared with citrate treated controls and this effect gradually declined. A similar pattern of analgesic effect was observed after each of the four consecutive daily i.pl. administrations of PF-0868087, that may potentially suggest a lack of pharmacological tolerance after peripheral administration of PF-0868087. Interestingly, 24 hours after the administration of PF-0868087 the effect of higher dose 0.3mg/paw was observed, that may potentially suggest long duration of the anti-nociceptive effect after peripheral administration when compared with controls and systemic administration (Figure 6.4, D).Indeed, significant difference in mechanical hypersensitivity was also, seen with the lowest dose of PF-0868087 (0.003 mg/paw) (6.6±0.7) when compared to the control group (2.9±0.3) (Figure 6.4 F; P<0.0001, n=6).

6.4.3 Systemic and peripheral administrations of PF-0868087 did not attenuate thermal hypersensitivity in neuropathic mice

The analgesic efficacy of systemic i.p. and localised peripheral i.pl. administration of PF-0868087 was also assessed by measuring the paw withdrawal latency in response to thermal stimuli using the Hargreaves test in CCI mice. Systemic administration of PF-0868087 did not produced effect on heat hypersensitivity in comparison with control vehicle group(Figure 6.4 A,C and E; P>0.05, n=6). Also, no significant difference in heat hypersensitivity was observed at any doses of PF-0868087 tested after localised peripheral administration (Figure 6.4 B, D and E P>0.05, n=6). AUC summarizing time-course effect of i.p. injection of PF-0868087 and/or vehicle on the thermal withdrawal threshold measured with the Hargreaves test: after i.p. administration of PF-0868087 1 mg/kg: 106.3 ±9.3 ,3 mg/kg: 103.8 ±6.7, 10 mg/kg: 112.8 ±7.1, and vehicle: 116.3±7.1 and after i.pl. administration of PF-0868087 0.003 mg/paw: 57.6 ±2.4, 0.03 mg/paw: 55.3 ±2.5, 0.3 mg/paw: 56.1 ±2, and vehicle: 56.3±2.5.

6.4.4 Systemic and peripheral administrations of PF-0868087 did not attenuate mechanical hypersensitivity in sham controls

The effectiveness of systemic (i.p.) and peripheral (i.pl.) administration of PF-0868087 was assessed by measuring the paw withdrawal threshold in response to mechanical stimuli using von Frey filaments in sham control-mice 7 days after surgery. Both route of administration of PF-0868087 (i.p.: 10mg/kg or i.pl.: 0.3mg/paw) showed a lack of change in paw withdrawal threshold in ipsilateral paws. AUC summarizing time-course effect of i.p. injection of PF-0868087 or vehicle on the mechanical hypersensitivity threshold measured with von Frey filaments: after i.p. administration of PF-0868087, 10mg/kg: 14.7±0.7, and vehicle: 14.9±0.8; and after i.pl. administration of PF-0868087, 0.3 mg/paw: 12.6 ±0.6, and vehicle: 14.2 ±0.5 (Figure 6.6; $P>0.05$, n=6).

6.4.5 Systemic and peripheral administrations of PF-0868087 did not attenuate thermal hypersensitivity in sham controls

The effectiveness of systemic (i.p.) and peripheral (i.pl.) administration of PF-086087 was assessed by measuring the paw withdrawal latency in response to thermal stimuli using the Hargreaves test in sham control-mice 7 days after surgery. Both route of administration of PF-086087 (i.p.: 10mg/kg or i.pl.: 0.3mg/paw) showed a lack of change in paw withdrawal threshold in ipsilateral paws. AUC summarizing time-course effect of i.p. and i.pl. injection of PF-0868087 and/or vehicle on the thermal withdrawal threshold measured with the Hargreaves test: after i.p. administration of PF-0868087, 10mg/kg: 83.0±20.0, and vehicle: 80.0±14.7; and after i.pl. administration of PF-0868087, 0.3 mg/paw: 94.1 ±2.9, and vehicle : 102.4 ±2.3 (Figure 6.7;C and D $P>0.05$, n=6).

6.4.6 Peripheral administration of PF-0868087 did not change mechanical and thermal hypersensitivity in contralateral (uninjured) paw in both neuropathic and sham mice

As illustrated in Figures 6.8 and 6.9, the contralateral hind paws (uninjured) did not exhibit statistically significant changes of withdrawal threshold for both mechanical and thermal hypersensitivity when compared with the threshold before surgery. The analgesic efficacy of localised peripheral (i.pl.) administration of PF-0868087 was assessed by measuring the paw withdrawal threshold in response to mechanical and thermal stimulus using von Frey filaments and Hargreaves test. No significant difference in both mechanical and thermal hypersensitivity was observed at any doses of PF-

0868087 tested after localised peripheral i.pl. administration in both CCI and sham control mice. AUC summarizing time-course effect of i.pl. injection of PF-0868087 and/or vehicle on the mechanical and thermal withdrawal threshold: after i.pl. administration of PF-0868087 on the mechanical 0.3mg/paw: 17.0±1.0, and vehicle: 18.1±0.8; and after i.pl. administration of PF-0868087 on thermal, 0.3 mg/paw: 92.7 ±3.3, and vehicle : 92.1 ±2.8 (Figures 6.8;C and 6.9;C respectively ; P>0.05, n=6).

6.4.7 Peripheral administration of PF-0868087 to the area distal from the injury did not attenuate mechanical and thermal hypersensitivity in neuropathic mice.

To confirm that the anti-nociceptive effect produced after i.pl. administration of PF-0868087 may be mediated by the involvement of peripheral H_3R present in the area of injury, we administrated PF-0868087 (0.3 mg/kg, s.c.) into the skin fold on the animal's neck that represents an area distal from the actual injury. The selected dose was the highest most effective i.pl. dose tested in CCI mice. The effectiveness of s.c. administration of PF-0868087 was assessed by measuring the paw withdrawal latency in response to mechanical and thermal stimuli using von Frey filaments and Hargreaves test, respectively, in CCI mice 7 days after the surgery. No change in paw withdrawal threshold and latency to mechanical or thermal stimuli were observed in CCI mice after s.c. administration of PF-0868087 in both ipsi and contralateral paws. AUC summarizing time-course effect of s.c. injection of PF-0868087 and/or vehicle on the mechanical and thermal withdrawal threshold: after s.c. administration of PF-0868087 on the mechanical 0.3mg/paw ipsi: 2.4±0.2, and vehicle: 2.7 ±0.3; and contra: 18.5±0.8, and vehicle: 19.4±0.6) and after s.c. administration of PF-0868087 on thermal, 0.3 mg/paw, ipsi: 45.1 ±1.9, and vehicle : 50.0 ±1.9 (Figure 6.10;A-D P>0.05, n=6).

6.4.8 Peripheral administration of a H_3R agonist immepip blocked the anti-nociceptive effect of PF-0868087 in the ipsilateral hind paw in neuropathic mice

In order to determine if the anti-nociceptive effects produced after administration of PF-0868087 were mediated *via* H_3Rs pathway the effect of PF-0868087 were examined in conjunction with immepip (H_3R agonist). As illustrated in Figure 6.11., local peripheral administration of immepip (100µg, i.pl.) significantly reversed the anti-nociceptive effect produced by systemically administered PF-0086087 (10 m/kg i.p.) in CCI mice (Figure 6.11, P<0.0001, n=5-6). After systemic administration the mechanical hypersensitivity in CCI mice was significantly reduced with PF-0868087 at 10 mg/kg

however local i.pl. administration of immepip (100µg /paw) 30 before i.p. injection of PF-0868087 (10 mg/kg) blocked the anti-nociceptive effect produced by PF-0868087. No significant difference in thermal hypersensitivity was observed after i.p. admistration of PF-0868087 either alone or when administrated after immepip (100µg /paw, n = 5-6). AUC summarizing time-course effect of i.p. injection of PF-0868087 and/or vehicle on the mechanical and thermal withdrawal threshold either alone or when administrated after immepip. Mechanical threshold: after i.p. administration of PF-0868087, 10mg/kg: 6.3±0.7, and vehicle: 1.4±0.2; and after i.pl. administration of immepip before PF-0868087, 0.3 mg/paw: 2.1±0.3, and vehicle: 1.4±0.2) (Figure 11;A, n=5-6). Thermal threshold: after i.p. administration of PF-0868087, 10mg/kg: 51.4±2.8, and vehicle: 44.6±2.8; and after i.pl. administration of immepip before PF-0868087, 0.3 mg/paw: 47.6 ±2.3, and vehicle: 44.6±2.8 (Figure 11;B, n=5-6).

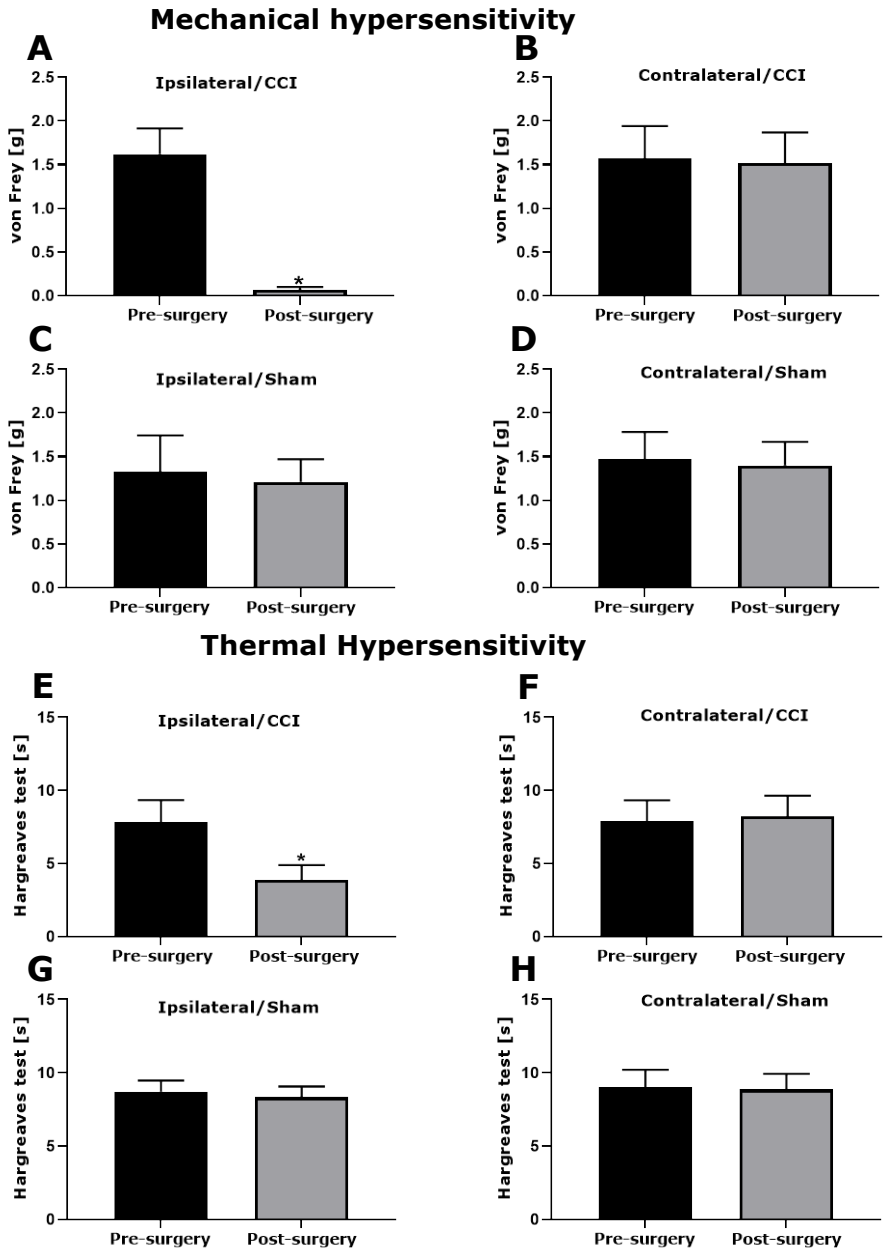


Figure 6.3 Chronic constriction injury (CCI) produced significant change in response to mechanical and thermal stimuli in mice. The von Frey and Hargreaves tests were used to measure the mechanical paw withdrawal threshold and thermal paw withdrawal latency in pre-CCI and post-CCI mice. (A and E) On day 7 after CCI surgery, mice showed an enhanced response to mechanical and thermal stimulation only in the ipsilateral part (injured hind paw) compared with their values prior the surgery. Experiment II: On day 7 after sham surgery, mice did not show any different response to mechanical (C) and thermal (G) stimulation in the lateral part of the injured hind paw (ipsi) compared with their values prior the surgery. The non-injured hind paw (contra) did not show any significant response in both CCI and sham to mechanical (B and D, respectively) and thermal (F and H) stimulation compared with their values prior to the surgery. Data are presented as mean ± SEM, n = 5-6 in each group, *P<0.05 * (two-tailed unpaired t-test).

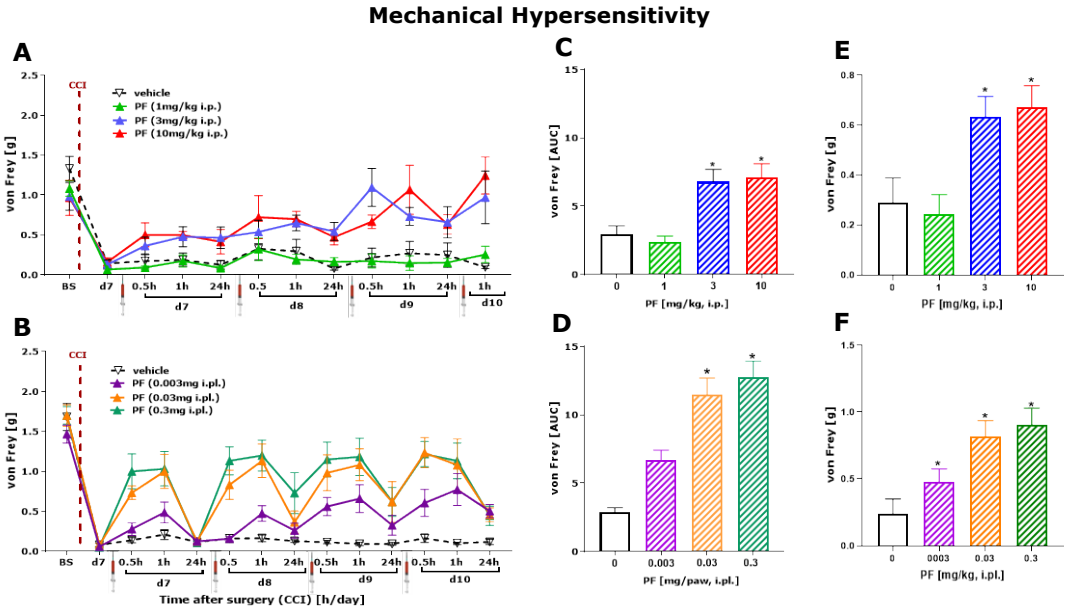


Figure 6.4 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of PF0086087 significantly attenuated mechanical hypersensitivity in the chronic constriction injury (CCI) model of neuropathic pain in mice. (A, B) Time-course effect of i.p. injection of PF0086087 (PF, 1-10 mg/kg, **A)** and i.pl. injection of PF0086087 (PF, 0.003-0.3 mg/paw, **B)** administered into the plantar surface of the injured paw, or vehicle (citrate buffer, A-B), on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of PF0086087 was assessed 0.5, 1 and 24 h after each of four consecutives i.p. (A) or i.pl. (B) injections. The syringe indicates each of four i.p. (A) or i.pl. (B) injections of PF0086087. Data are presented as means ± S.E.M, n=6 in each group. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. (E) and (F) Comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test. The asterisk (*) denotes significance *vs.* vehicle control animals; *p<0.05 (one-way ANOVA, followed by Bonferroni's comparison post-hoc test, E and F repeated measure ANOVA followed by Bonferroni's test.).

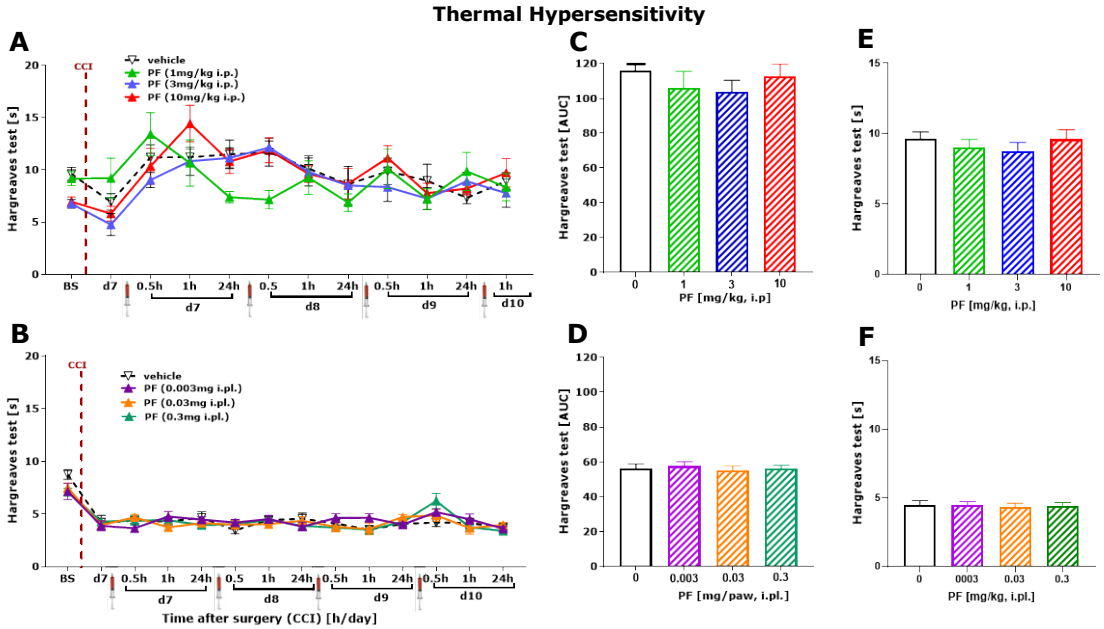


Figure 6.5 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of PF0086087 did not attenuate thermal hypersensitivity in the chronic constriction injury (CCI) model of neuropathic pain in mice. (A, B) Time-course effect of i.p. injection of PF0086087 (PF, 1-10 mg/kg, A) and i.pl. injection of PF0086087 (PF, 0.003-0.3 mg/paw, B) administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of PF0086087 was assessed 0.5-24 h after each of four consecutives i.p. (A) or i.pl. (B) injections. The syringe indicates each of four i.p. (A) or i.pl. (B) injections of PF0086087. Data are presented as means ± S.E.M, n=6-8 in each group. (C) The area under the curve (AUC) summarizing measurements in A. (D) The area under the curve (AUC) summarizing measurements in B. (E) and (F) Comparison of the groups with repeated measure one-way ANOVA followed by Bonferroni's test. Lack of significant changes *vs.* vehicle control animals; *p>0.05 (one-way ANOVA, followed by Bonferroni's comparison post-hoc test and E, F repeated measure ANOVA followed by Bonferroni's test.).

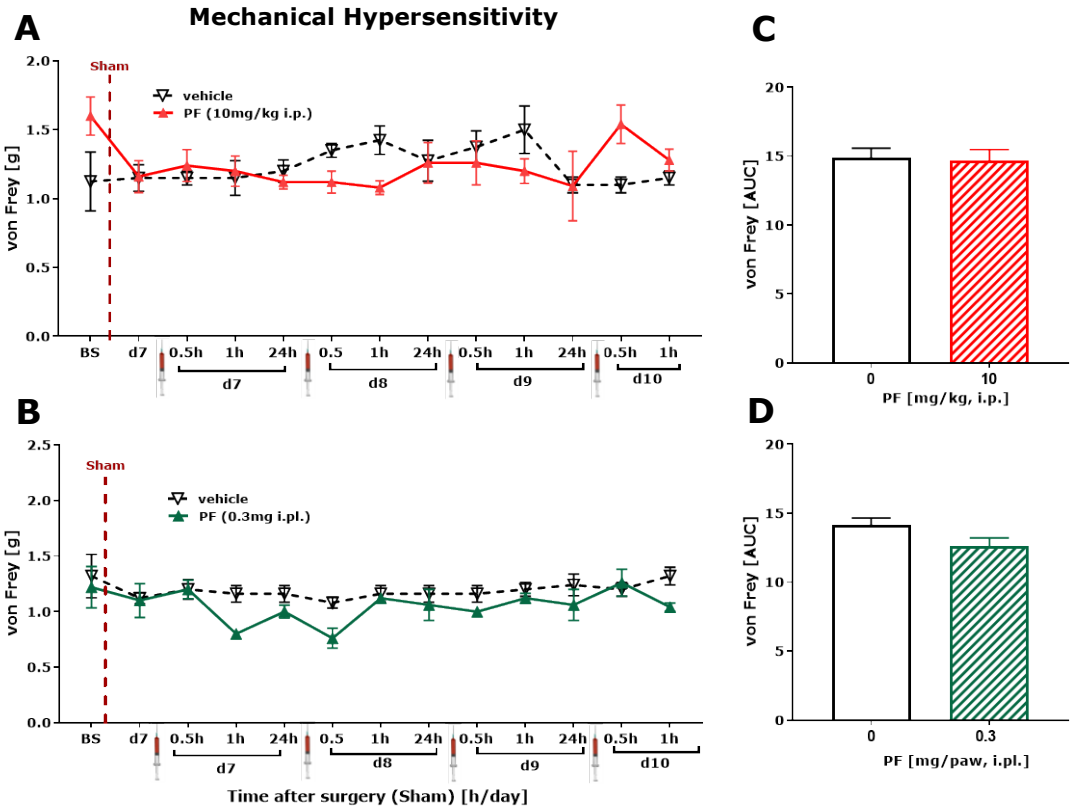


Figure 6.6 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of PF0086087 did not attenuate overall mechanical hypersensitivity in sham mice. (A, B) Time-course effect of i.p. injection of PF0086087 (PF, 10 mg/kg, **A)** and i.pl. injection of PF0086087 (PF, -0.3 mg/paw, **B)** administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of PF0086087 was assessed 0.5,1 and 24 h after each of four consecutives i.p. (A) or i.pl. (B) injections. The syringe indicates each of four i.p. (A) or i.pl. (B) injections of PF0086087. Data are presented as means ± S.E.M **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. Lack of significant changes *vs.* vehicle control animals; *p>0.05 (two-tailed unpaired t-test).

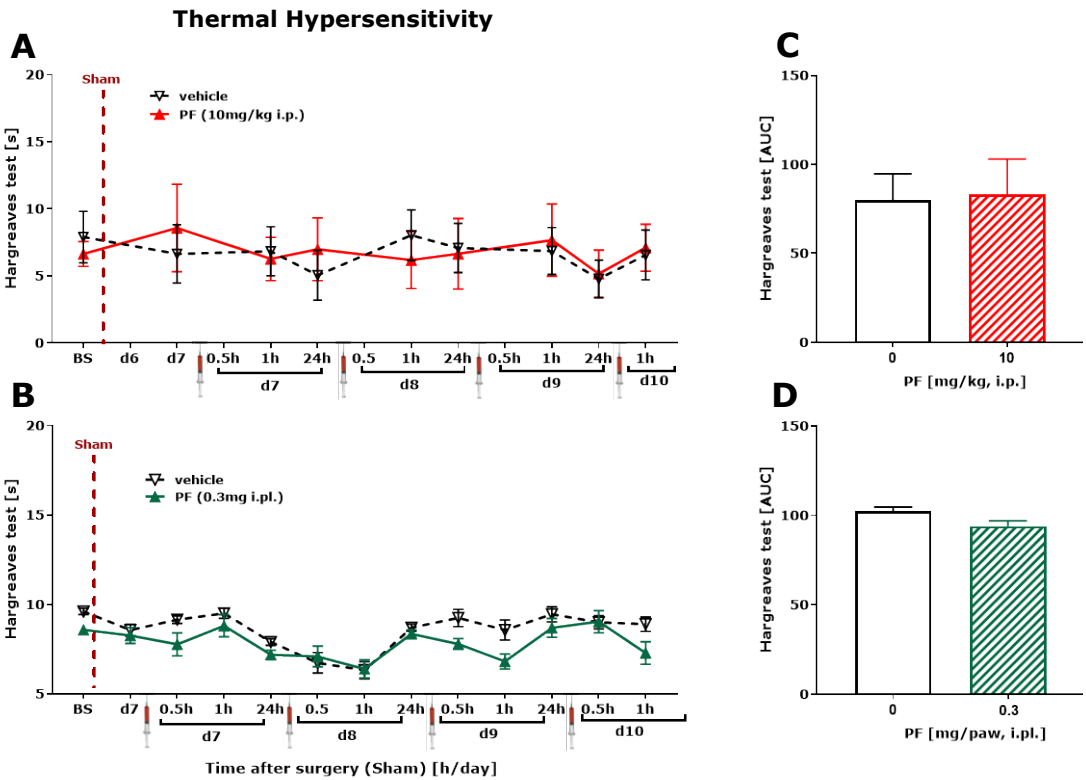


Figure 6.7 Chronic systemic intraperitoneal (i.p.) and peripheral intraplantar (i.pl.) administration of PF0086087 did not attenuate overall thermal hypersensitivity in sham mice. (A, B) Time-course effect of i.p. injection of PF0086087 (PF, 10 mg/kg, A) and i.pl. injection of PF0086087 (PF, -0.3 mg/paw, **B)** administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of PF0086087 was assessed 0.5-24 h after each of four consecutives i.p. (A) or i.pl. (B) injections. The syringe indicates each of four i.p. (A) or i.pl. (B) injections of PF0086087. Data are presented as means ± S.E.M. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. Lack of significant changes *vs.* vehicle control animals; *p>0.05 (two-tailed unpaired t-test).

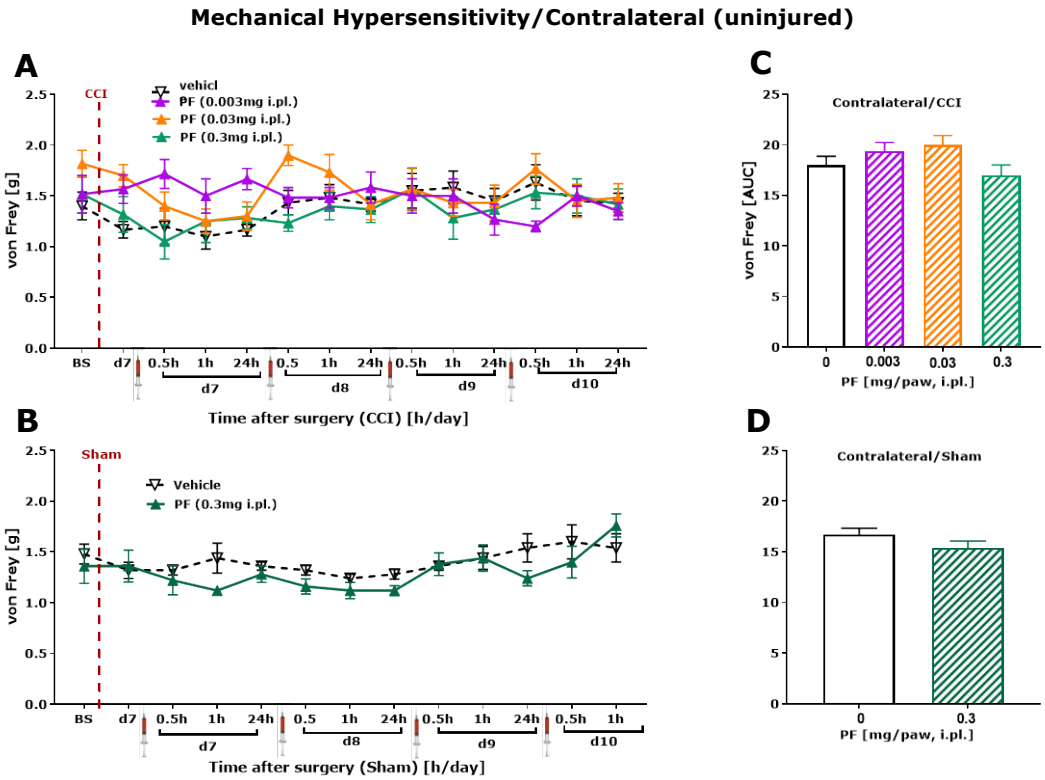


Figure 6.8 Chronic peripheral intraplantar (i.pl.) administration of PF0086087 did not attenuate overall mechanical hypersensitivity in both CCI and sham mice at the contralateral plantar surface of the hind paw. (A, B) Time-course effect of i.pl. injection of PF0086087 (PF, 0.003-0.3 mg/paw, A) and (PF, 0.3 mg/paw, **B)** administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the mechanical withdrawal threshold measured with von Frey filaments n the contralateral hind paw (uninjured paw) . The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of PF0086087 was assessed 0.5-24 h after each of four consecutives i.pl. (A) injections. The syringe indicates each of four i.pl. injections of PF0086087. Data are presented as means ± S.E.M, n=6-8 in each group. **(C)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in B. Lack of significant changes vs. vehicle control animals; *p>0.05 for A,C (one-way ANOVA, followed by Bonferroni's comparison post-hoc test) and for B,D (two-tailed unpaired t-test).

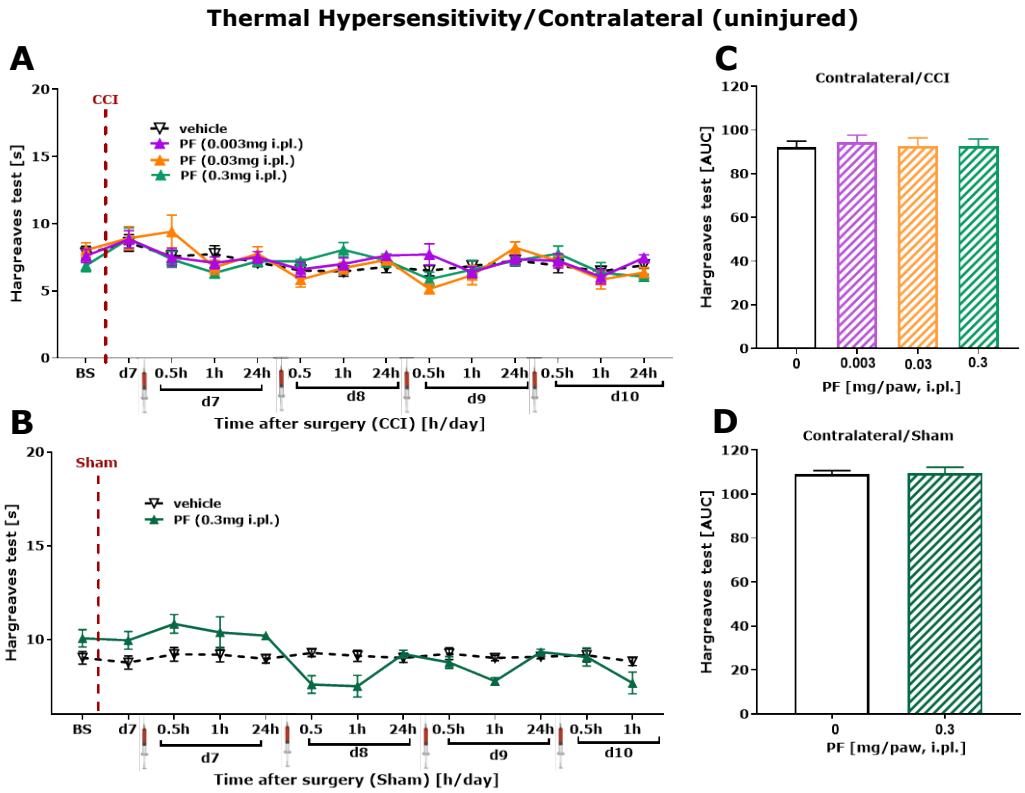


Figure 6.9 Chronic peripheral intraplantar (i.pl.) administration of PF0086087 did not attenuate overall thermal hypersensitivity in both CCI and sham mice at the contralateral plantar surface of the hind paw. (A, B) Time-course effect of i.pl. injection of PF0086087 (PF, 0.003-0.3 mg/paw, A) and (PF, 0.3 mg/paw, B) administered into the plantar surface of the injured paw, or vehicle (saline, A-B), on the mechanical withdrawal threshold measured with von Frey filaments n the contralateral hind paw (uninjured paw) . The measurements were assessed before injury as basal pain threshold (BS) and then 7 days following the injury (d7). The effect of PF0086087 was assessed 0.5-24 h after each of four consecutives i.pl. (A) injections. The syringe indicates each of four i.pl. injections of PF0086087. Data are presented as means ± S.E.M, n=6-8 in each group. (C) The area under the curve (AUC) summarizing measurements in A. (D) The area under the curve (AUC) summarizing measurements in B. Lack of significant changes vs. vehicle control animals; *p>0.05 (one-way ANOVA, followed by Bonferroni's comparison post-hoc test) and (two-tailed unpaired t-test).

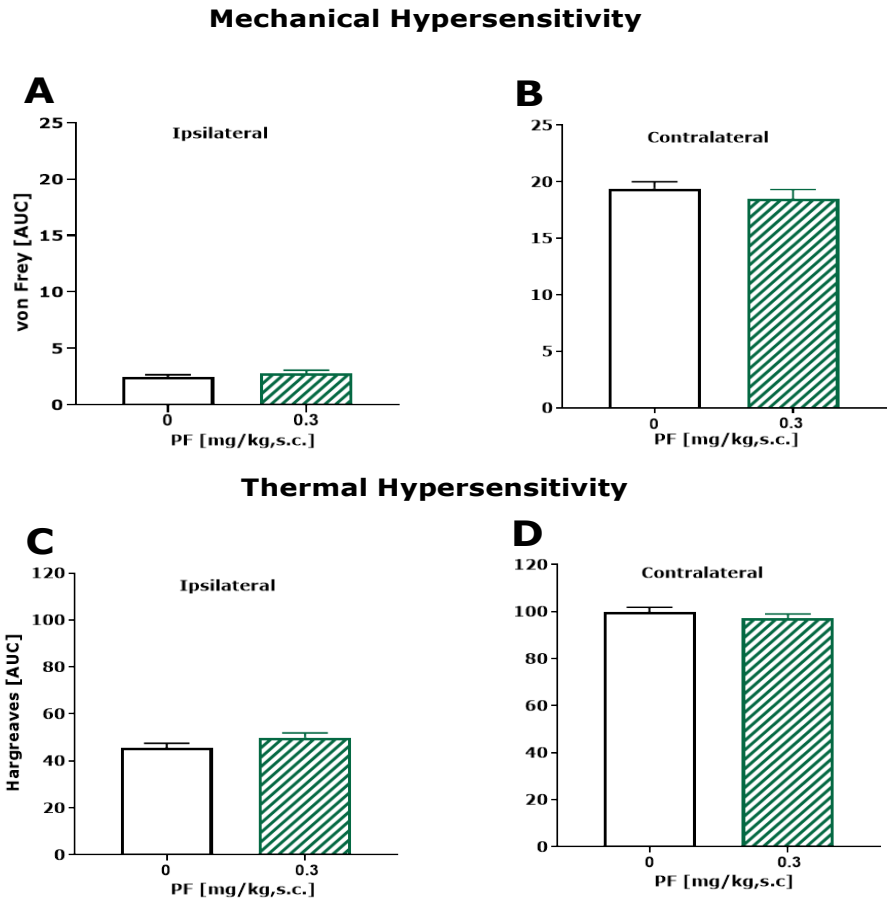


Figure 6.10 Chronic peripheral subcutaneous (s.c) administration of PF0086087 did not attenuate thermal hypersensitivity in the chronic constriction injury (CCI) model in mice. (A and B) AUC represent the effects of S.C injection administered or vehicle of PF0086087 (0.3 mg/kg) or vehicle in CCI mice on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were taken on the ipsilateral or contralateral paw (A and B respectively). (C and D) AUC represent the effects of local S.C injection of PF0086087 (0.3 mg/kg) or vehicle in CCI mice on the thermal withdrawal threshold measured with the Hargreaves test. The measurements were assessed before injury as the basal pain threshold (BS) and then 6 days post-injury. The effect of PF0086087 was assessed at; 1 h post-administration of the four-consecutive s.c injections. *P<0.05 vs vehicle control animals. Lack of significant changes vs. vehicle control animals; *p>0.05 (two-tailed unpaired t-test).

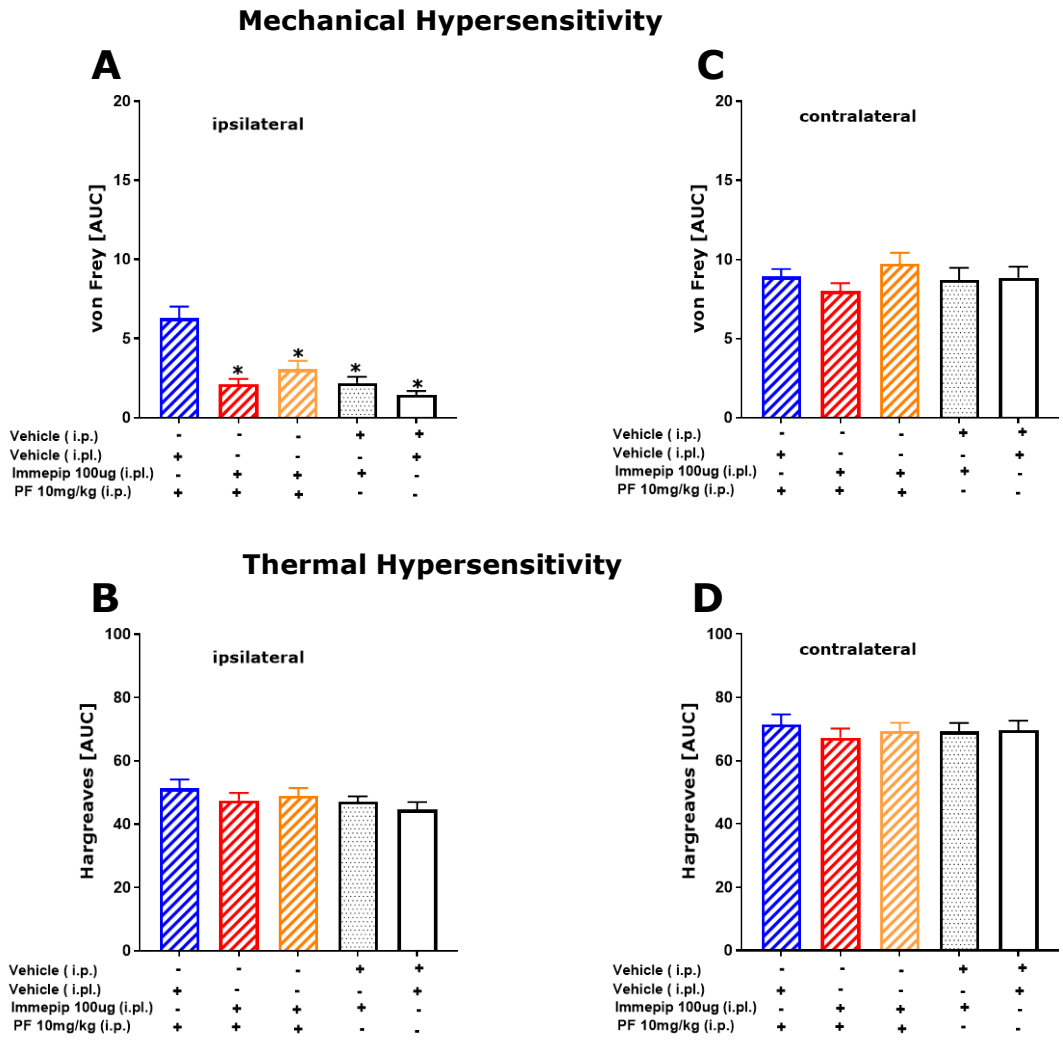


Figure 6.11 Chronic peripheral intraplantar (i.pl.) administration of immepip (H_3R agonist) blocked the anti-nociceptive effect of PF0086087 in the in the ipsilateral hind paw in chronic constriction injury (CCI) model of neuropathic pain in mice. The area under the curve (AUC) summarizing time-course effect of systemic intraperitoneal (i.p.) injection of PF0086087 (PF, 10 mg/kg, n = 5) a lone and in combination with peripheral intraplantar (i.pl.) injection of immepip (100µg /paw, n = 6), or vehicle (saline, n = 5), on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were taken on the ipsilateral or contralateral paw (A and C respectively). **(D-F)** The area under the curve (AUC) summarizing time-course effect of systemic intraperitoneal (i.p.) injection of PF0086087 (PF, 10 mg/kg, n = 5) a lone and in combination with peripheral intraplantar (i.pl.) injection of immepip (100µg /paw, n = 6), or vehicle (saline, n = 5), on the thermal withdrawal threshold measured with the Hargreaves test. The measurements were taken on the ipsilateral or contralateral paw (B and D respectively) (*detailed data not shown*). The asterisk (*) denotes significance *vs.* systemic intraperitoneal (i.p.) injection of PF0086087; *p<0.05 (two-tailed unpaired t-test).

6.5 Discussion

The results of the studies in this Chapter, demonstrate the analgesic effects of a novel CNS-sparing H_3R antagonist, PF-0868087 after systemic (i.p.) or local peripheral (i.pl.) administration in a pre-clinical model of neuropathic pain in mice that are mediated predominantly by peripheral H_3Rs. These findings may provide a solid foundation for further investigation of the involvement of peripheral H_3Rs in the mediation of anti-nociceptive effects in chronic pain. Our findings are consistent with previous data showing that targeting of H_3R using selective antagonists reduced symptoms of neuropathic pain (Medhurst *et al.*, 2008; Cowart *et al.*, 2012; Popiolek-Barczyk *et al.*, 2018). However, the advantage of the data shown in this Chapter is that we have revealed the evidence for the involvement of peripheral H_3R in neuropathic pain for the first-time.

As previously highlighted, neuropathic pain is the result of lesions or diseases of the somatosensory nervous system which leads to significant changes in the structure and function of this system. Neuropathic pain is largely resistant to available medications and the underlying mechanisms are still poorly understood. Many hypotheses have been proposed to explain underlying mechanisms, but none appear to account for the various elements of neuropathic pain complexity (Vilela-Filho *et al.*, 2014). Literature evidence supports involvement of pro-inflammatory mediators such as cytokines and chemokines in chronic neuropathic pain processing. According to pathophysiology of neuropathic pain, it appears that the development of this type of pain is principally due to the development of a local inflammatory response that occurs in the affected area, which results in the release of a wide range of mediators (Clatworthy et al., 1995). Histamine is one of the key mediators accountable for the development of hypersensitivity following nerve injury (Tatarkiewicz et al., 2019). It has been shown previously by Foreman and Jordan (1984) that when histamine is released at the site of injury, it binds to a specific receptor located in the primary afferent nerve ending, causing local sensitization of nerve fibers and the generation of an action potential across the fibers. This increases the stimulation rate along the nerve fibers (Zimmermann, 2001; Gopalsamy et al., 2019). Therefore, targeting of HRs, particularly H_3R by using a selective antagonist/inverse agonist seems to control the

neuronal depolarization activities induced by histamine.

H_3R consists of several functional isoforms expressed within the ascending and descending pain pathways at CNS and PNS (dermal tissues, DRG, spinal, supraspinal and in various brain areas) that are essential for the processing of nociceptive information. In fact, a potential role for H_3R in pain processing has been suggested by several previous studies. Therefore, this specific type of receptor could provide treatment options for various types of chronic pain including neuropathic pain (Moreno-Delgado et al., 2006; Moreno-Delgado et al., 2006; Cannon et al., 2007; Medhurst et al., 2008; Obara et al 2020).

In the present study, the development of mechanical and thermal hypersensitivity in ipsilateral side of CCI mice was confirmed by von Frey monofilaments and Hargreaves test respectively. This study adds further evidence that CCI-induced both allodynia and hyperalgesia *in vivo* (De Vry et al., 2004; Espinosa-Juárez et al., 2017; Limcharoen et al., 2020; Kwankaew et al., 2021). Moreover, the contralateral paw was also tested but no changes in mechanical or thermal thresholds were detected after surgery. The present research findings seem to be consistent with previous research which found contralateral paw not affected after CCI surgery (Holdridge and Cahill, 2007; Limcharoen et al., 2020; Kwankaew et al., 2021; Zhang et al., 2021). On the other hand, this finding appears to contradict previous studies which reported that peripheral nerve injury-induced contralateral mechanical allodynia (Mansikka et al., 2004) and induced inflammation as well as changes in the release of inflammatory mediators (Levine et al., 1987; Kleinschnitz et al., 2005; Pitcher et al., 2013) and alteration of expression of different types of receptors and channels (Huang et al., 2007; Dubový et al., 2010). This conflict in the findings could be due to many reasons such as the type of preclinical pain model used, the nociceptive stimulus was chosen, and the affinity and selectivity for the histamine receptors, dose, pharmacokinetics, routes of administration of the tested drugs and the timeline of the experiment.

We also observed that systemic and localized peripheral administration of PF-0868087, H_3R antagonist, reduced symptoms associated with neuropathic pain. This PF-0868087-induced effect highlights the importance of H_3R for the maintenance of neuropathic pain symptoms in the periphery. This possibility is consistent with other previous studies,

Hsieh et al (2010) which showed that the systemic administration of the potent and selective H_3R antagonist; GSK189254, produced dose-dependent efficacy against mechanical and cold hyperalgesia associated with neuropathic pain (Hsieh et al., 2010). In addition, novel histamine H_3R antagonist, GSK207040 significantly inhibited capsaicin-induced secondary allodynia in rats, (Medhurst et al., 2007). The same research group in 2008 found that repetitive oral administration of GSK334429, selective H_3R decreased paw withdrawal thresholds in rats with neuropathic pain (Medhurst et al., 2008). Similarly, Hsieh et al., (2010) found that H_3R antagonists have been reported to be effective in reducing allodynia and hyperalgesia, in *in vivo* models of both neuropathic and inflammatory pain (Hsieh et al., 2010). A subsequent study by Popiolek-Barczyk et al., in 2018 found that E-162, an H_3R antagonist, after systemic (i.p) administration reduced symptoms of NeP in CCI mice (Popiolek-Barczyk et al., 2018). Thus, consistently with the previously reported results (Medhurst et al., 2007; Popiolek-Barczyk et al., 2018), our data demonstrate that both acute and chronic systemic (i.p.) and local (i.pl.) treatment with H_3R antagonist reduced mechanical hypersensitivity after chronic constriction nerve injury. Also, we found that the anti-nociceptive effect of PF-0868087 was most potent after localised peripheral administration; the lowest anti-nociceptive peripheral effective dose was almost 2-fold lower in comparison to the lowest effective systemic dose. Importantly, preclinical research previously published have shown that PF-0868087 does not penetrate the blood-brain barrier (Lunn *et al.*, 2012) and based on that it seems likely that PF-0868087 is blocking peripheral H_3R after both systemic and local administration. However, the localized i.pl. the application shows a more potent and longer duration of action by small dose compared to systemic i.p. administration which highlights the important targeting of peripheral H_3R.

This finding stands in contrast to some other experimental studies, in which H_3R agonists (not antagonists) have anti-inflammatory and anti-nociceptive properties (Rouleau et al., 1997; Cannon et al., 2003). In 2007, Cannon and co-workers demonstrated that stimulation of H_3R by immepip reduced the formalin-evoked pain behaviour, and this anti-nociceptive effect was reversed by administration of the thioperamide, H_3R antagonist (Cannon et al., 2007). Interestingly, in 2007, Huang et al. published a paper in which they described that thioperamide, H_3R antagonist have distinct effects on the

nociceptive threshold depending on the route of administration since central administration helped to increase pain tolerance while systemic application decreased it (Huang et al., 2007). These opposite results seem to show that H_3R agonists might be beneficial for the treatment of certain types of pain and H_3R antagonists for another pain type (Cannon and Hough, 2005; Hsieh et al., 2010). It appears that several factors may contribute to the effect of the drug and may lead to different responses such as preclinical pain models used, different routes of administration, and differences in the affinity and selectivity to the receptors, which significantly affect the biological reaction for given H_3R agonists and antagonists (Ghamari et al., 2019; Obara et al., 2020). However, following previous studies, H_3R antagonists seem relatively more advantageous than an H_3R agonist in terms of anti-nociceptive effects and can be promising agents for the treatment of several types of pain, especially neuropathic pain (Ghamari et al., 2019; Obara et al., 2020). However, further research is required to make the role of the central and peripheral H_3R receptors in neuropathic pain clearer.

Interestingly, however, while mechanical hypersensitivity was blocked by local and systemic administered PF-0868087, heat hypersensitivity remained unaffected. This finding may be linked to the research of Cannon et al., (2007) and Lunn et al., (2012), who suggested that heat hypersensitivity is considered as clue for the peripheral sensitization and mediated predominantly by unmyelinated C-fibres, which do not express H_3R. This specific anti-nociceptive effect of PF-0868087 in neuropathic pain model may suggest that PF-0868087 binds to H_3R located on A-fibres that conduct tactile sensation (Cannon et al., 2007; Lawson, 2002). Thus, PF-0868087, by selective blocking of the H_3Rs, reduced the sensitivity of H3-positive A-fibres resulting in a reduced input to the dorsal horn, supporting the possible role for H_3R in the alteration of peripheral and central sensitization. Potential involvement of H_3R on A-fibres in the regulation of nociception can be also linked to the studies of Medhurst et al., (2007), where H_3R antagonist GSK207040 blocked secondary mechanical allodynia. To our knowledge, this is the first study to demonstrate the effect of peripheral H_3R antagonists on CCI-induced mechanical allodynia, supporting the theory that blocking H_3R receptors may improve mechanical allodynia in neuropathic pain patients. Interesting to note that, while mechanical hypersensitivity was inhibited by PF-0868087 administered peripherally,

heat hypersensitivity was not affected by this treatment. This specific anti-nociceptive effect of PF-0868087 may indicate that the H_3R is involved in pain transmission from the PNS to the CNS since PF-0868087 works by blocking H_3R. Given that both pharmacological and genetic alterations in the expression of the H_3R have demonstrated its significance and specificity for mechanical hypersensitivity (Cannon et al., 2003; Wei et al., 2016). Moreover, anatomical studies have also confirmed the presence of H_3R receptors on A-fibers that transmit pain signals (Cannon et al., 2007; Lawson, 2002). Based on this finding, it is possible to speculate that PF-0868087, by blocking H_3R, decreased the activity of H_3R-positive A-fibres, resulting in a diminished input to the dorsal horn, thereby supporting the possibility that H_3R receptors are involved in the modulation of central sensitization. thermal hypersensitivity, on the other hand, is regarded as a sign of the peripheral sensitization of C-fibers, which do not express H_3R receptors and therefore do not cause heat hypersensitivity (Cannon et al., 2007), and that may explain that heat hypersensitivity was not affected by PF-0868087 treatment. In contrast to our results, only one research stated a considerable rise of thermal threshold in the spared nerve injury model of neuropathic pain after treatment with pitolisant, selective H_3R inverse agonists. Zhang et al., (2012) explained this unexpected effect produced by pitolisant was because the dose of the drug used was five times higher than its clinically relevant dose (Zhang et al., 2012). Also, pharmacological evidence indicates that the mechanisms that cause this unpredictable effect of pitolisant are specifically related to the TRPV1-mediated pathway without the involvement of H_3Rs (Zhang et al., 2012).

In addition, results with immepip, H_3R agonist, treatment strongly suggest the possibility that peripheral H_3Rs can modulate neuropathic pain. Mechanical allodynia was attenuated by a systemic dose of PF-0868087 in mice subjected to CCI of the sciatic nerve. This analgesic effect was blocked by immepip administrated peripherally into the injured hind paw thus, suggesting the contribution of peripheral H_3Rs in the intervention of this anti-nociceptive effect. Following the mechanisms of injury-induced hypersensitivity, it is likely that PF-0868087 targeted exclusively a subpopulation of A-delta fibres countering particularly to mechanical stimulations where H_3R is exclusively expressed (Cannon et al., 2007; Medhurst et al., 2008; Hough and Rice, 2011).

Finally, to confirm that these anti-nociceptive effects of PF-0086087 are mediated by peripheral receptors localized at the site of injury, we found that s.c. administration (the selected dose was the highest effective i.pl. dose tested in CCI mice) did not induce any changes in the pain threshold of ipsi and contralateral paw which maintained values similar to those measured in control animals. These results may additionally support our claim that the effects of PF-0086087 were mediated by a local peripheral receptor, at the site of injury.

The outcomes from the current *in vivo* behavioral studies, assessing the effect of potent and selective H_3R antagonist, PF-0868087 has demonstrated that this subtype of histamine receptor is involved in the transmission and perception of pain during neuropathy. The results of the present study provide the first evidence for the analgesic effects of PF-0868087 upon neuropathic pain-related mechanical hypersensitivity, and this emphasizes the importance of the histamine system in the modulation of peripheral neuropathies. Generally, local administration of analgesics may have several advantages over systemic administration, including the ability to use lower doses, in that way minimizing side effects, drug interaction and potential toxicity (Bagshaw et al., 2015). Moreover, according to current concepts in pain medicine, which emphasize the importance of an individualized and mechanism-based approach to pain management (Müller-Schwefe et al., 2017; Kocot-Kopska et al., 2021). Using local PF-0868087 is in the same line with this approach since it only targets the underlying molecular/cellular mechanisms in the periphery of the body.

In summary, PF-0868087 is a novel H_3R antagonist with a promising preclinical profile and has structural relation with PF-3654746 which was already used in clinical studies for allergic rhinitis (Liu, 2010; Lunn et al., 2012). However, our tested compound PF-0868087 has a more beneficial effect than PF-3654746 (which can cross the blood-brain barrier and cause insomnia as a major side effect). Furthermore, PF-0868087 had excellent solubility and demonstrated good pharmacokinetic profiles. All these advantages made PF-0868087 a promising drug for use in clinical trials shortly, however further preclinical animal studies are needed before their use in humans.

CHAPTER 7

Interaction between H_3R and mTORC1 pathway in neuropathic pain

7.1 Introduction

In the previous chapter, the significance of H_3R in the neuropathic pain has been demonstrated based on animal studies using a neuropathic pain model in mice and PF-0868087, a selective and peripherally acting/centrally sparing antagonist targeting H_3R. While a significant analgesic effect was produced by PF-0868087 in neuropathic pain in mice. The cellular and molecular mechanisms underlying the antinociceptive effect of PF-0868087 and antagonism at H_3R that results in blocking pain is not fully understood.

It has been demonstrated that H_3R is highly expressed on neuronal cells located in the CNS, where it exert negative feedback regulation on the release of wide range of neurotransmitters including neuropeptides, GABA, glutamate and biogenic amines like norepinephrine, acetylcholine and dopamine (Schlicker et al., 1999; Blandina et al., 1996; Jang et al., 2001; Cowart et al., 2012; Garduño-Torres et al., 2007; Nieto-Alamilla et al., 2016; Flores-Clemente et al., 2021). H_3R on histaminergic neuron control of histamine release *via* an autoregulatory mechanism (Cowart et al., 2012). Therefore, stimulation of H_3R inhibits histamine and other neurotransmitters release from synaptic vesicles of neurons, while blocking this receptor increases histamine and other neurotransmitters release (Arrang et al., 1983; Cowart et al., 2012). Several of potent and selective H_3R antagonists have been identified and tested in pre-clinical studies, many of them have progressed to the clinical testing stage (Leurs et al., 2011; Cowart et al., 2012). A number of studies have found that H_3R antagonist are efficient in the control of variety of pre-clinical models, including schizophrenia, sleep disorders, cognition, and obesity (Schwartz, 2011; Kumar et al., 2019). However, there are very few publications on the antinociception effects of H_3R antagonists, indicating that the mechanism underlying these compounds is not completely understood. However, several H_3R antagonists have been shown to be effective in different pre-clinical pain studies such as diabetic neuropathic pain, osteoarthritic, inflammatory pain, surgical neuropathic

pain (Medhurst et al., 2007; Medhurst et al., , 2008; Hsieh et al., 2010). In support of this, studies in rats have shown that GSK-207040 and GSK-334329 have anti-nociceptive efficacy by reducing capsaicin-induced secondary hyperalgesia (Medhurst et al., 2007). Moreover, Medhurst et al., (2008) found that GSK-189254 and GSK-334329 after oral administration had antinociceptive effect in CCI-induced neuropathic pain (Medhurst et al., 2008). In our current research, as shown previous Chapter, we found that PF-0868087, a selective and peripherally acting/centrally sparing H_3R antagonist produced significant antinociceptive effect in neuropathic mice. All these studies indicate that H_3R has a critical roles in the development and modulation of histamine-mediated pain. However, the mechanism by which H_3R antagonists exert their antinociceptive effect is unknown.

Interestingly, in 2014 Yan and colleagues, found that the inhibition of H_3R caused inhibition of the Akt/GSK-3β pathway, which subsequently inhibited mTORC1 activity (Yan et al., 2014). This was an interesting observation since H_3R with multiple isoforms can target a variety of signal transduction pathways in both PNS and CNS. Since 1999, the cloning of the *H_3R* gene by Lovenberg et al, (1999), allowed thorough studies on the molecular properties of this type of receptor and showed that H_3R can activate numerous signal transduction pathways at once (Herrera-Zúñiga *et al.*, 2020; Bongers *et al.*, 2007), including adenylate cyclase/cAMP/PKA signaling pathway (Leurs et al., 2005), as well as the inhibition of Na^+/H^+ exchanger pathway and inhibition of cAMP/ K^+ /Ca^{2+} mobilization pathway (Leurs et al. 2005; Bongers et al., 2007). Additionally, in primary cultured rat cortical neurons, H_3R was shown to stimulate the Akt/GSK-3β signaling pathway *via* both agonist-dependent and agonist-independent (constitutive) activity (Lovenberg et al. 1999; Bongers et al., 2007; Yan et al., 2014). Previous studies indicated that Akt/GSK-3β signalling pathway is associated with numerous pathological conditions and diseases, such as convulsions, neuropathic pain, obesity, cerebral ischaemic injury, and neurological disorders (Bongers et al., 2007; Yan et al., 2014; Bhowmik et al., 2014). Interestingly, it has been demonstrated that Inhibition of H_3R protect against neurotoxicity induced by NMDA in primary cultures of cerebrocortical neurons (Dai et al., 2007). Therefore, most recently, it has been hypothesised that the inhibition of H_3R activity has neuroprotective effects *via* the inhibition of Akt/GSK-3β

/mTOR pathway (Yan et al., 2014; Bongers et al., 2007).

Based on our studies and other pre-clinical evidence, it can be proposed that H_3R may have a potential role in neuropathic pain transmission that is potentially associated with a subsequent inhibition of mTOR pathway. Interestingly, mTOR was identified as a relatively novel target to effectively control chronic pain, and in particular neuropathic pain (Geranton et al., 2009; Wang et al., 2016; Obara et al., 2011). As described in the first Chapter, mTOR is one of a serine threonine protein kinase. The mTOR assembles into two structurally and functionally different protein complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). In general, mTORC1 serve as a gate that controls translation most of the proteins *via* controlling the activation of specific downstream effectors such as ribosomal S6 proteins (S6), p70 ribosomal protein S6 kinase (p70 S6K) and eukaryotic initiation factor 4E-binding protein 4E-BP (Liang et al., 2013; Hay and Sonenberg, 2004). The mTORC1 and its downstream effectors play vital roles in the regulation of protein synthesis, growth, learning, memory, and neuronal plasticity (Banko *et al.*, 2005; Costa-Mattioli *et al.*, 2009; Duan *et al.*, 2018). In mammals, mTORC1 and its downstream signalling proteins are expressed in various parts of the CNS such as hippocampus (Tang et al., 2002), ventral tegmental area (Mazei-Robison et al., 2011), cortex (Um et al., 2019; Kwon et al., 2017) dorsal horn neurons of the spinal cord (Geranton et al., 2009; Liang et al., 2013; Xu et al., 2010), and also in PNS such as dorsal root ganglion and myelinated A peripheral fibres (Geranton et al., 2009; Jiménez-Díaz et al., 2008; Obara et al., 2011; Xu et al., 2010).

Activation of the mTORC1 pathway leads to phosphorylation of the downstream signalling proteins 4EBP, eIF4B, S6 kinase (S6K1), and consequently lead to changes in protein translations (Hay and Sonenberg, 2004; Xu et al., 2010). It has been widely proven that mTORC1 and downstream effectors are involved in the development of a wide range of disorders such as metabolic diseases (Szwed, Kim and Jacinto, 2021; Laplante and Sabatini, 2012), chronic inflammation(Soltani et al., 2018), pruritus (Obara et al., 2015), chronic neurodegenerative disease (Wang et al., 2016), cancer (Pópulo, Lopes and Soares, 2012; Tian, Li and Zhang, 2019) and many types of chronic pain such as inflammatory, and neuropathic pain (Obara et al., 2011; Wang et al., 2016; Liang et

al., 2013; Lisi et al., 2015). In the case of neuropathic pain, multiple lines of evidence support the idea that peripheral nerve injury prompted both molecular and biochemical neuroplasticity changes in the somatosensory nervous system, including ascending/descending pain transmission pathways; these changes lead to the development of neuropathic pain (Goswami et al., 2016; Han, 2017; Bosnar Puretić and Demarin, 2012). The present study mainly focussed on the possible interactions between H_3R and two molecules of mTORC1 and tested the hypothesis proposed in a previous study by Yan et al. (2014) using a cerebral ischaemic injury. Figure 7.1 represents the proposed model for the interactions between H_3R and mTORC1. The proposed mechanism of this inhibition is that H_3R antagonism prevents CLIC4-H_3R binding; this process subsequently ~~cause~~ causes an inhibition of PI3K/Akt/GSK-3β/mTORC1 signalling pathway (Yan et al., 2014). Interestingly, many studies proposed that mTORC1 and its downstream effectors might have been implicated in the development and regulate of many types of chronic pain one of them being neuropathic pain (Lisi *et al.*, 2015; Obara *et al.*, 2011).

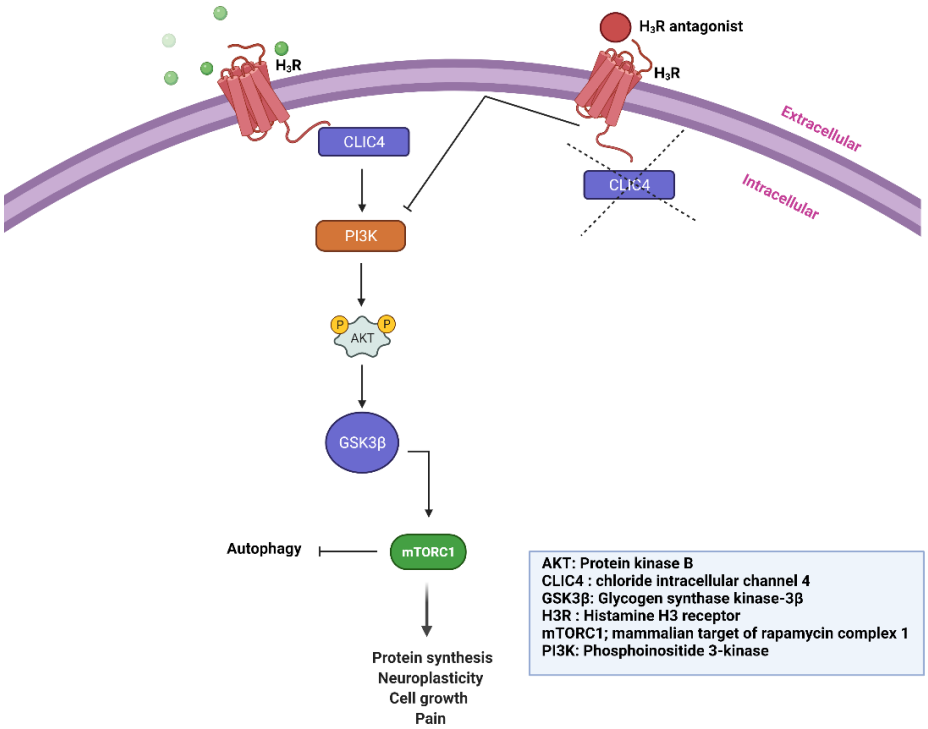


Figure 7.1 Schematic pathway of potential interaction between H_3R and mTORC1. The interaction between H_3R and H_3R antagonist leads to releases of CLIC4 from the binding site on H_3R. This process subsequently caused an inhibition of PI3K/Akt/GSK-3β/mTORC1 signalling pathway that significantly resulted in decreasing of cell growth, protein synthesis and attenuation of pain perception (adapted from Yan et al., 2014).

Therefore, in this chapter, it was hypothesized that mTORC1 and its downstream effectors are involved in the regulation of analgesic effect produced by PF -0868087 in the spinal dorsal horn and sciatic nerve of neuropathic mice. Western blotting analysis technique was used to test whether that the mechanism proposed earlier by Yan et al. (2014) may in fact be involved in the analgesia resulting from the antagonism at H_3R.

As shown in Chapter 5, the antinociceptive effect of H_3R, PF-0868087 was observed in the CCI model of neuropathic pain and here we propose that this effect might be *via* histamine-independent mechanisms which can be identified as a novel approach for the future therapeutic development of pain control, especially since H_3R antagonist and mTOR inhibitors have been used successfully to treat excessive daytime sleepiness and used as antineoplastic agents, respectively.

7.2 Material and Methods

7.2.1 Subject

Experiments in this chapter were carried out using adult male C57BL/6J mice (8 weeks of age; 20–25 g; Charles River Laboratories, Harlow, UK) as previously described in Chapter two (Section 2.2). Animals underwent surgery for a model of experimental mononeuropathy, a unilateral chronic constriction injury to the sciatic nerve (CCI; Section 2.5.1). On the 4th day of treatment (1-2 h after the last morning PF-0868087 injection) mice were euthanised by cervical dislocation and samples of the spinal cord and sciatic nerves were collected. Tissue preparation, immunoblotting, loading and running the gel, antibody labelling and visualisation of interested proteins as previously described in Chapter two (Section 2.7). Data was collected from both the ipsilateral and contralateral side. A schematic diagram of this experiment is shown in Figure 7.2.

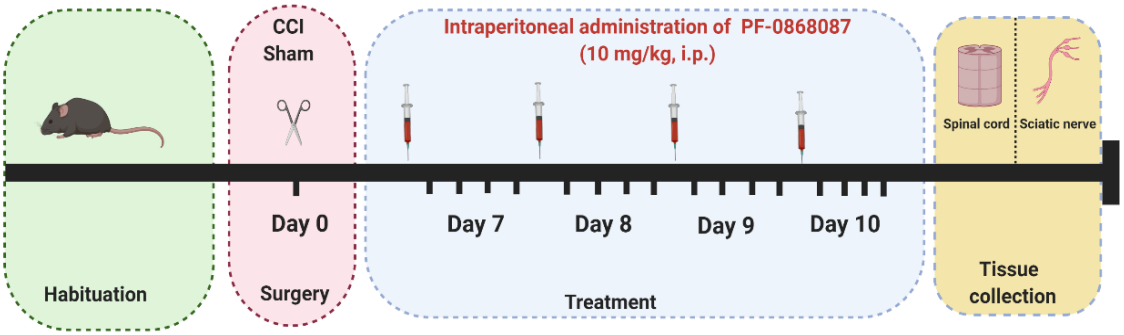


Figure 7.2 A schematic illustration of experiment timeline. Represents the chronic administration of PF-0868087 (10 mg/kg, i.p.) (once daily for 4 consecutive days). The blue line represents the injection of PF-0868087 or citrate buffer as control. The final step involved tissue collection for Western blot analysis.

7.2.2 Preparation and administration of drugs

PF-0868087 was provided by Ziarco Pharma (UK) as a pure crystalline powder and stored in adequate conditions (protected from humidity and light) in the refrigerator between 2 and 4°C. For all administrations, PF-0868087 was prepared immediately before injections and dissolved in a vehicle (sterile 0.1M citrate buffer solution) at required concentrations as described below.

Systemic intraperitoneal (i.p) administration. Mice were weighed and randomized to receive either PF-0868087 or vehicle (citrate buffer); they were injected i.p. with PF-0868087 at 1, 3, and 10 mg per kg body weight or vehicle (0.1M citrate buffer) solution without PF-0868087 as a control group . PF-0868087/vehicle was administered once every 24 hours on days 7, 8, 9 and 10 post induction of neuropathic pain. The animals received a total of four i.p. administrations of PF-0868087/vehicle.

All other chemicals including, sodium citrate dihydrate, hydrochloric acid, citric acid, and sodium hydroxide, were obtained from Sigma-Aldrich Chemical Co, UK. Drugs were injected in a volume of 100 µl for i.p. administration.

7.2.3 Neuropathic pain model

7.2.3.1 Induction of neuropathic pain

The method for the induction of neuropathic pain is described in detail in Chapter 2.

7.2.4 Design of the experiment

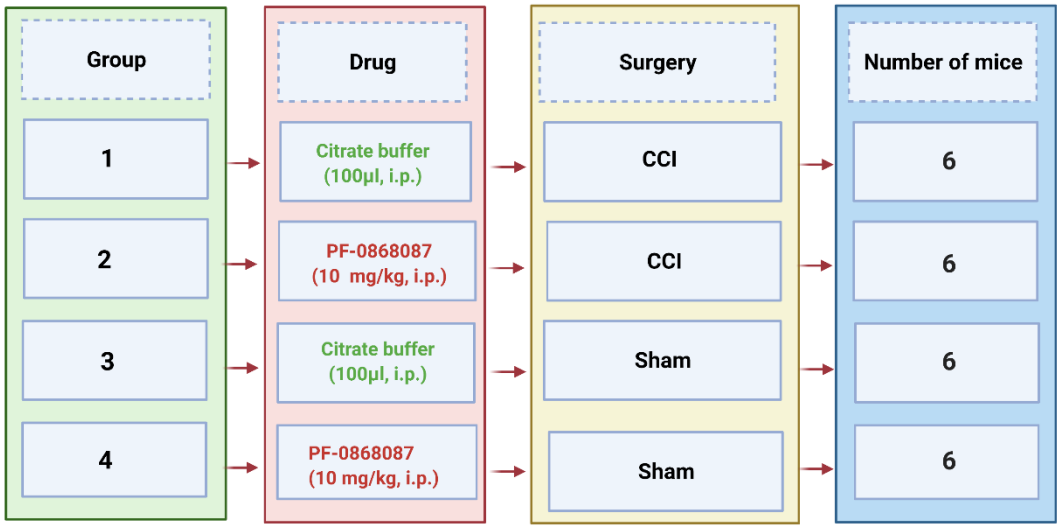
To study the role H_3R antagonist PF-0868087 on the activity of mTORC1 pathway, Western blot (WB) analyses was performed on the spinal cord and sciatic nerve sample collected from both CCI and sham mice subjected to the treatment with PF-0868087.

Mice were divided into 4 experimental groups as indicated in Table 7.1. Group 1 (n= 6) consisted of CCI mice receiving PF-0868087 (10 mg/kg, i.p.) one dose per day at same time for 4 consecutive days. Group 2 was the control group (n= 6) consisting of CCI mice receiving citrate buffer under the same condition as the 1st group. Group 3 (n= 6)

consisted of sham mice receiving PF-0868087 (10 mg/kg, i.p.) one dose per day at same time for 4 consecutive days, and the last group 4 control group (n= 6) consisted of sham mice receiving citrate buffer under the same condition as the 3rd group (controls). On the 4th day of treatment (1-2 h after the last morning PF-0868087 injection) mice were euthanised by cervical dislocation and samples of the spinal cord and sciatic nerves were collected.

The Western blotting analysis was carried out with soluble fractions of spinal cord, specifically the lumber area of L4-L6 and sciatic nerve (ipsi and contra).The ubiquitous housekeeping enzyme glyceraldehyde-3-phosphate dehy¬drogenase (GAPDH) was used as a control, for protein level normalisation. For more details about the processing of the samples and antibody detection using western blotting ChemiDoc MP imaging system see Chapter 2.

Table 7.1 Experimental groups



CCI: Chronic constriction injury, **i.p.:** intraperitoneal and.
CCI and sham mice were randomly assigned into 4 groups (n=6), received different doses of PF /vehicle(citrate buffer) control as following **(Group 1)** CCI mice received i.p. injection of vehicle (citrate buffer) in a volume of 100µL; **(Groups 2)** CCI mice received i.p. injection of PF-0868087 (10 mg/kg); **(Group 3)** Sham mice received i.p. injection of vehicle(citrate buffer) in a volume of 100 µL; **(Group 7)** Sham mice received i.p. injection of PF-0868087 (10 mg/kg). Tissue was taken 1-2 hours after the last morning PF-0868087 injection on day 10.

7.3 Statistical data analysis

All the data analysis and statistical comparisons were made using GraphPad PrismTM, (version 9.1.2) for Windows/OS (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Images were visualised using chemiluminescent substrate and BioRad Chemidoc. The optical density of the bands was then quantified using Image J (an open-source Java-based image processing program) to calculate the intensity of the bands. The results were expressed as a mean ratio of phosphor/total protein expression ± SEM graphs generated on a Graphpad Prism. The intensity of each antibody band was normalized to the intensity of the GAPDH signal, which was set to 100%. Each group included 5-6 mice. Unpaired student's t-test was used for comparisons between groups and the $p<0.05$ *vs* the respective control group was considered to be statistically significant.

7.4 Results

7.4.1 Systemic administration of PF-0868087 decreased p-mTORC1/mTOR ratio in the spinal cord of neuropathic mice.

As illustrated in Figure 6.3, systemic i.p. administration of PF-0868087 (10 mg/kg) once daily for 4 consecutive days (from day 7 to day 10 post CCI) significantly reduced the ratio of phosphorylated to total mTORC1 on the ipsilateral and contralateral side of the spinal cord in CCI mice (treatment effect ipsi: (60.2±16.9) $t_{(9)}$=2.8, P<0.05; treatment effect contra: (60.4±21.1) $t_{(10)}$=3.2, P<0.05, n=5-6) comparing with vehicle treated animals. However, the drug did not show a significant changes in the ratio of phosphorylated to total mTORC1 level in sham control mice (treatment effect ipsi: (66.1±29.0) $t_{(10)}$=1.2, P>0.05; treatment effect contra: (90.3±13.5) $t_{(10)}$=0.96, P>0.05 n=5-6).

7.4.2 Systemic administration of PF-0868087 decreased phosphorylation level of downstream targets of mTORC1 in spinal cord of neuropathic mice.

7.4.2.1 S6 ribosomal protein (S6RP)

As illustrated in Figure 7.4, systemic i.p. administration of PF-0868087 (10 mg/kg) once daily for 4 consecutive days (from day 7 to day 10 post CCI) significantly reduced the ratio of phosphorylated to total S6RP on the ipsilateral side of the spinal cord in the CCI mice (treatment effect ipsi: (66.0±9.4) $t_{(9)}$=3.4, P<0.05, n=5-6) comparing with vehicle treated animals. This effect was not observed on the contralateral side (treatment effect contra: (77.5±16.5) $t_{(10)}$=1.7, P>0.05, n=5-6), as well as PF-0868087 did not show a significant changes in the ratio of phosphorylated to total S6RP level in sham control mice (treatment effect ipsi: (83.6±16.6) $t_{(10)}$=1.7, P>0.05; treatment effect contra: (94.4±18.2) $t_{(10)}$=0.5, P>0.05, n=5-6).

7.4.2.2 Eukaryotic Translation Initiation Factor 4E Binding Protein 1 (4E-BP1)

As illustrated in Figure 7.5, systemic i.p. administration of PF-0868087 (10 mg/kg) once daily for 4 consecutive days (from day 7 to day 10 post CCI) significantly reduced the ratio of phosphorylated to total 4E-BP1 on the ipsilateral side of the spinal cord in the CCI mice (treatment effect ipsi: (75.1±9.1) $t_{(9)}$=2.7, P<0.05, n=5-6) comparing with vehicle treated animals. This effect was not observed on the contralateral side

(treatment effect contra: (88.6±11.5) $t_{(10)}$=0.7, P>0.05, n=5-6), as well as PF-0868087 did not show a significant changes in the ratio of phosphorylated to total 4E-BP1level in sham control mice (treatment effect ipsi: (76.0±26.5) $t_{(10)}$=1.6, P>0.05; treatment effect contra: (101.1±50.4) $t_{(10)}$=0.04, P>0.05, n = 5-6).

7.4.3.2 70 kDa ribosomal protein S6 kinase 1 (p70S6K)

As illustrated in Figure 7.6, systemic i.p. administration of PF-0868087 (10 mg/kg) once daily for 4 consecutive days (from day 7 to day 10 post CCI) reduced the ratio of phosphorylated to total p70S6K in the spinal cord in the CCI mice. However, a trend towards a reduction in the ratio of P-p70S6K / total p70S6K did not reach significance (treatment effect ipsi: (62.1±11.5) $t_{(9)}$=1.6, P>0.05; treatment effect contra: (84.8±8.7) $t_{(10)}$=1.3, P>0.05, n = 5-6) comparing with vehicle treated animals. The drug did not show a significant changes in the phosphorylation level of p70S6K in sham control mice (treatment effect ipsi: (75.6±28.2) $t_{(10)}$=1.5, P>0.05; treatment effect contra: (124.9±24.7) $t_{(10)}$=1.8, P>0.05, n=5-6).

7.4.3 Systemic administration of PF-0868087 decreased phosphorylation of downstream targets of mTORC1 in the sciatic nerve of neuropathic mice.

7.4.3.1 S6RP

As illustrated in Figure 7.7, systemic i.p. administration of PF-0868087 (10 mg/kg) once daily for 4 consecutive days (from day 7 to day 10 post CCI) significantly reduced phosphorylation of the S6RP on the ipsilateral side of the sciatic nerve in the CCI mice (treatment effect ipsi: (52.6±7.5) $t_{(10)}$=5.7, P<0.05) comparing with vehicle treated animals. This effect was not observed on the contralateral side (treatment effect contra: (105.8±41.5) $t_{(10)}$=0.3, P>0.05, n = 5-6). The drug also did not show a significant changes in the of the phosphorylation level of S6RP in sham control mice (treatment effect: ipsi: (82.6±15.3) $t_{(10)}$=1.2, P>0.05; contra: (82.7±17.9) $t_{(10)}$=1.5, P>0.05, n=5-6).

7.4.3.2 p70S6K

As illustrated in figure 6.8, systemic i.p. administration of PF-0868087 (10 mg/kg) once daily for 4 consecutive days (from day 7 to day 10 post CCI) reduced phosphorylation of the p70S6K (phospho- p70S6K) in the sciatic nerve in the CCI mice (treatment effect ipsi: (69.8±10.5) $t_{(9)}$=2.3, P>0.05) comparing with vehicle treated animals. This effect was not

observed on the contralateral side (treatment effect contra: (99.1±20.6) $t_{(10)}$=0.1, P>0.05, n = 5-6). The drug also did not show a significant changes in the phosphorylation level of p70S6K in sham control mice (treatment effect ipsi: (144.1±59.9) $t_{(10)}$=1.6, P>0.05; treatment effect contra: (82.7±17.9) $t_{(10)}$=2.2, P>0.05, n=5-6).

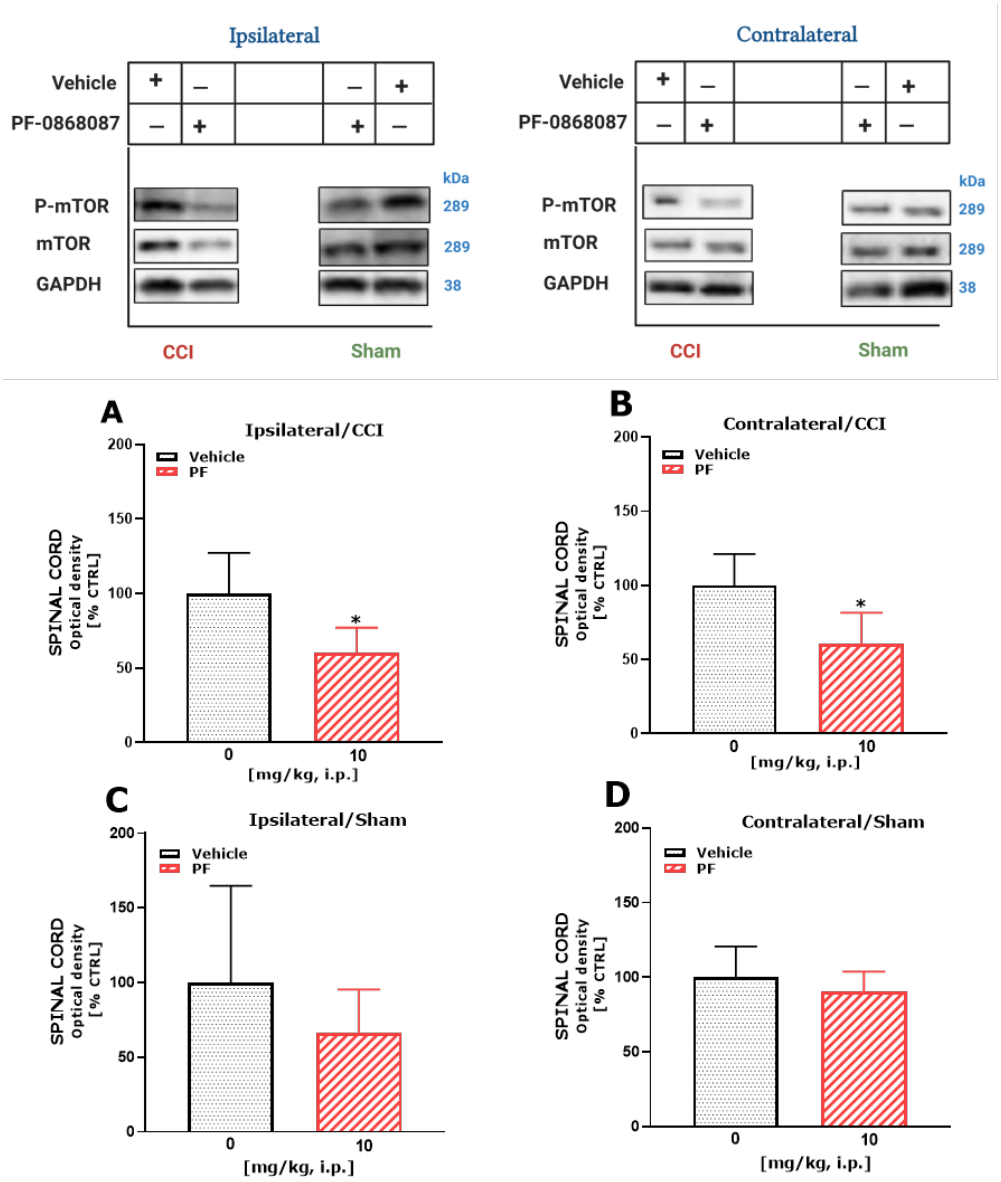


Figure 7.3 Chronic systemic intraperitoneal (i.p.) administration of PF-0868087 **significantly decreased the phosphorylation of mTORC1 in the spinal cord in the chronic constriction injury (CCI) model of neuropathic pain in mice. (A and B)** Effects of i.p. injection of PF-0868087 (10 mg/kg), or vehicle (V), on the level phospho-mTOR (P-mTOR) and total mTOR (A) ipsilateral and (B) contralateral side of the spinal cord. **(C, D)** represent the effect on the sham control mice. Mice received an i.p. injection of PF-0868087 or vehicle 1 dose every 24 hours at the same time for 4 constitutive days before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. n=5 to 6 per experimental group, each graph represents the phosphoprotein/total protein ratio, mean ± SEM values. The asterisk (*) denotes significance *vs* vehicle controls; * P<0.05 (unpaired Student t test). Representative Western immunoblots are shown above the graph bar.

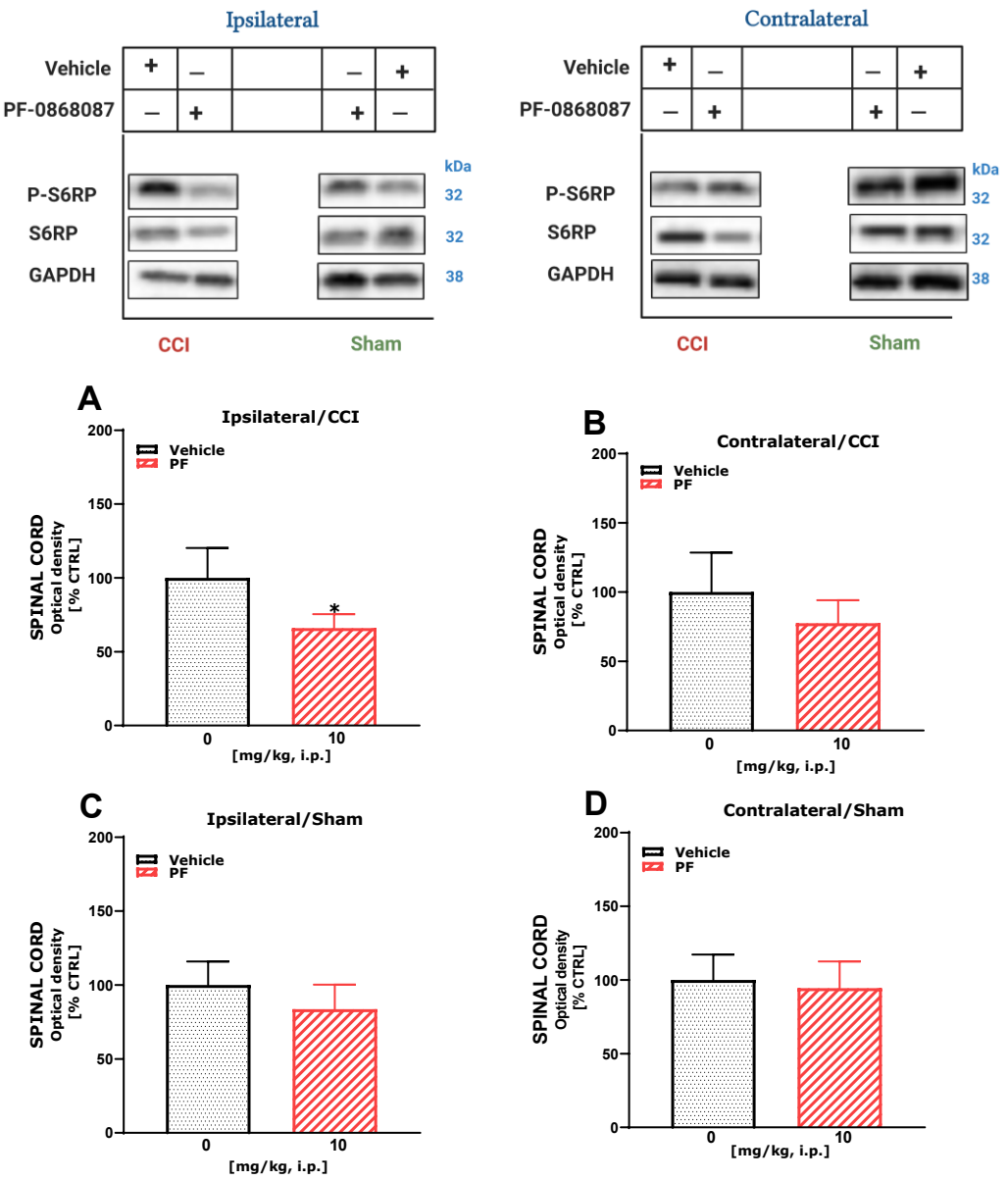


Figure 7.4 Chronic systemic i.p. administration of PF-0868087 significantly reduced the phosphorylation of downstream target of the mTORC1 in the spinal cord in the CCI model of neuropathic pain in mice. (A and B) Effects of i.p. injection of 8680871 (10 mg/kg), or vehicle (V), on the level phospho- S6RP (P- S6RP) and total S6RP **(A)** ipsilateral and **(B)** contralateral side of the spinal cord. **(C, D)** represent the effect on the sham control mice. Mice received an i.p. injection of PF-0868087 or vehicle 1 dose every 24 hours at the same time for 4 constitutive days before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. n=5 to 6 per experimental group, each graph represents the phosphoprotein/total protein ratio, mean ± SEM values. The asterisk (*) denotes significance *vs* vehicle controls; *P<0.05 (unpaired Student t-test). Representative Western immunoblots are shown above the graph bar.

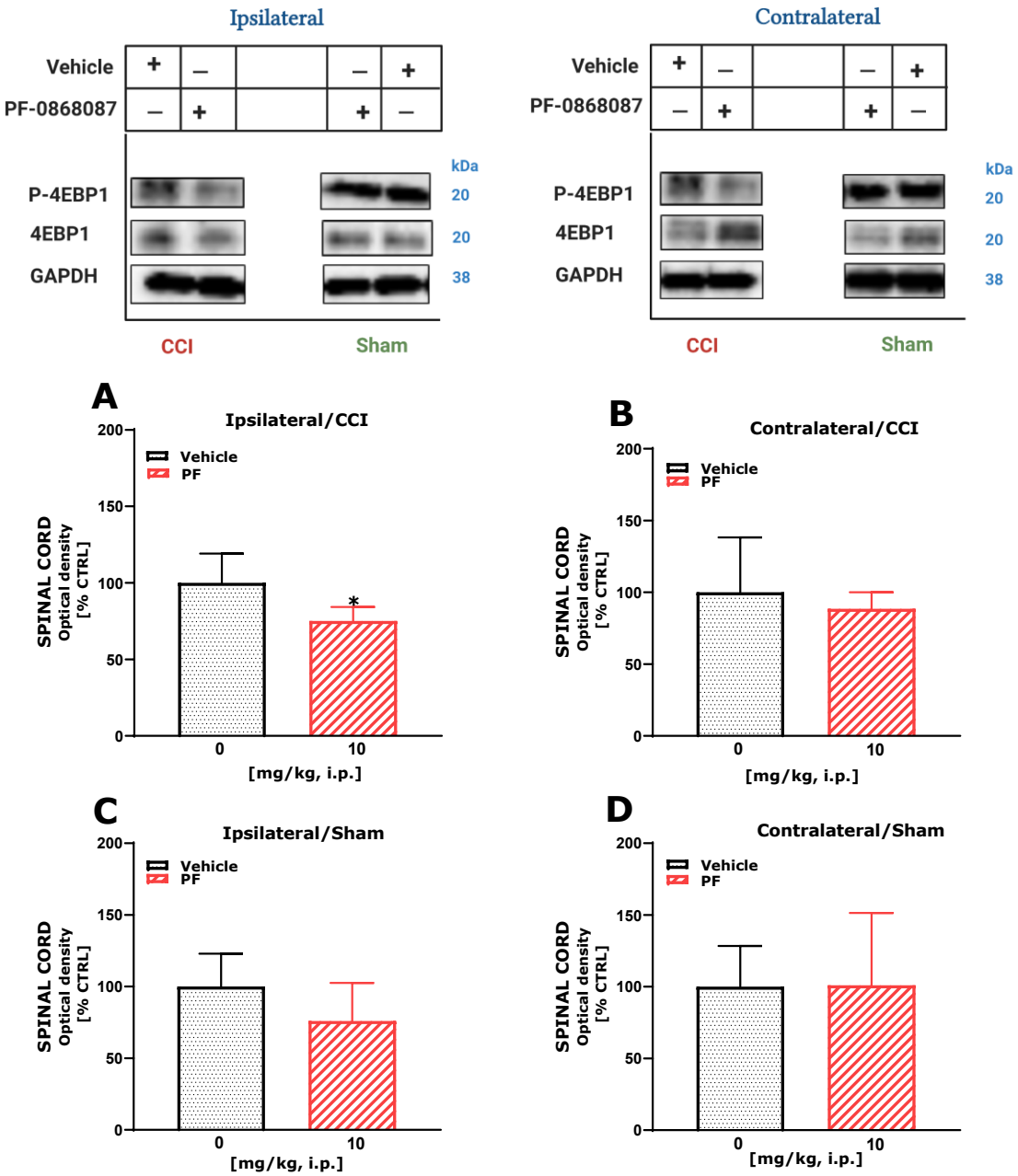


Figure 7.5 Chronic systemic i.p. administration of PF-0868087 significantly reduced the phosphorylation of the mTORC1 in the spinal cord in CCI model of neuropathic pain in mice. (A and B) Effects of i.p. injection of 8680871 (10 mg/kg), or vehicle (V), on the level phospho- 4E-BP1 (P-4E-BP1) and total 4E-BP1 **(A)** ipsilateral and **(B)** contralateral side of the spinal cord. **(C, D)** represent the effect on the sham control mice. Mice received an i.p. injection of PF-0868087 or vehicle 1 dose every 24 hours at the same time for 4 constitutive days before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. n=5 to 6 per experimental group, each graph represents the phosphoprotein/total protein ratio, mean ± SEM values. The asterisk (*) denotes significance *vs* vehicle controls; *P<0.05 (unpaired Student t-test). Representative Western immunoblots are shown above the graph bar.

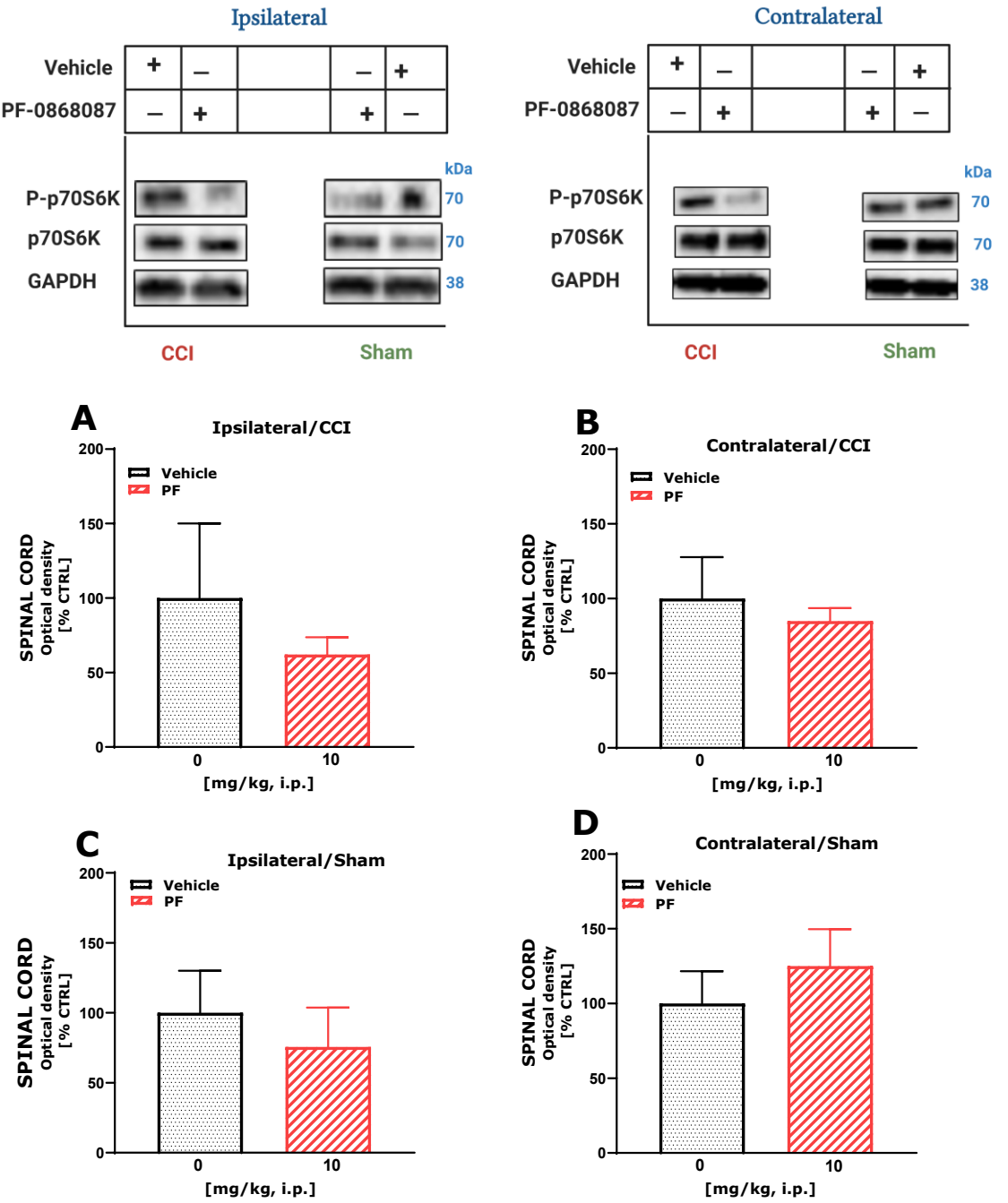


Figure 7.6 Chronic systemic i.p. administration of PF-0868087 decreased the phosphorylation of P70S6k in the spinal cord in the CCI model of neuropathic pain in mice. (A and B) Effects of i.p. injection of 8680871 (10 mg/kg), or vehicle (V), on the level phospho- and total P70S6K **(A)** ipsilateral and **(B)** contralateral side of the spinal cord. (**C, D)** represent the effect on the sham control mice. Mice received an i.p. injection of PF-0868087 or vehicle 1 dose every 24 hours at the same time for 4 constitutive days before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. n=5 to 6 per experimental group, each graph represents the phosphoprotein/total protein ratio, mean ± SEM values. The asterisk (*) denotes significance *vs* vehicle controls; *P<0.05 (unpaired Student t-test). Representative Western immunoblots are shown above the graph bar.

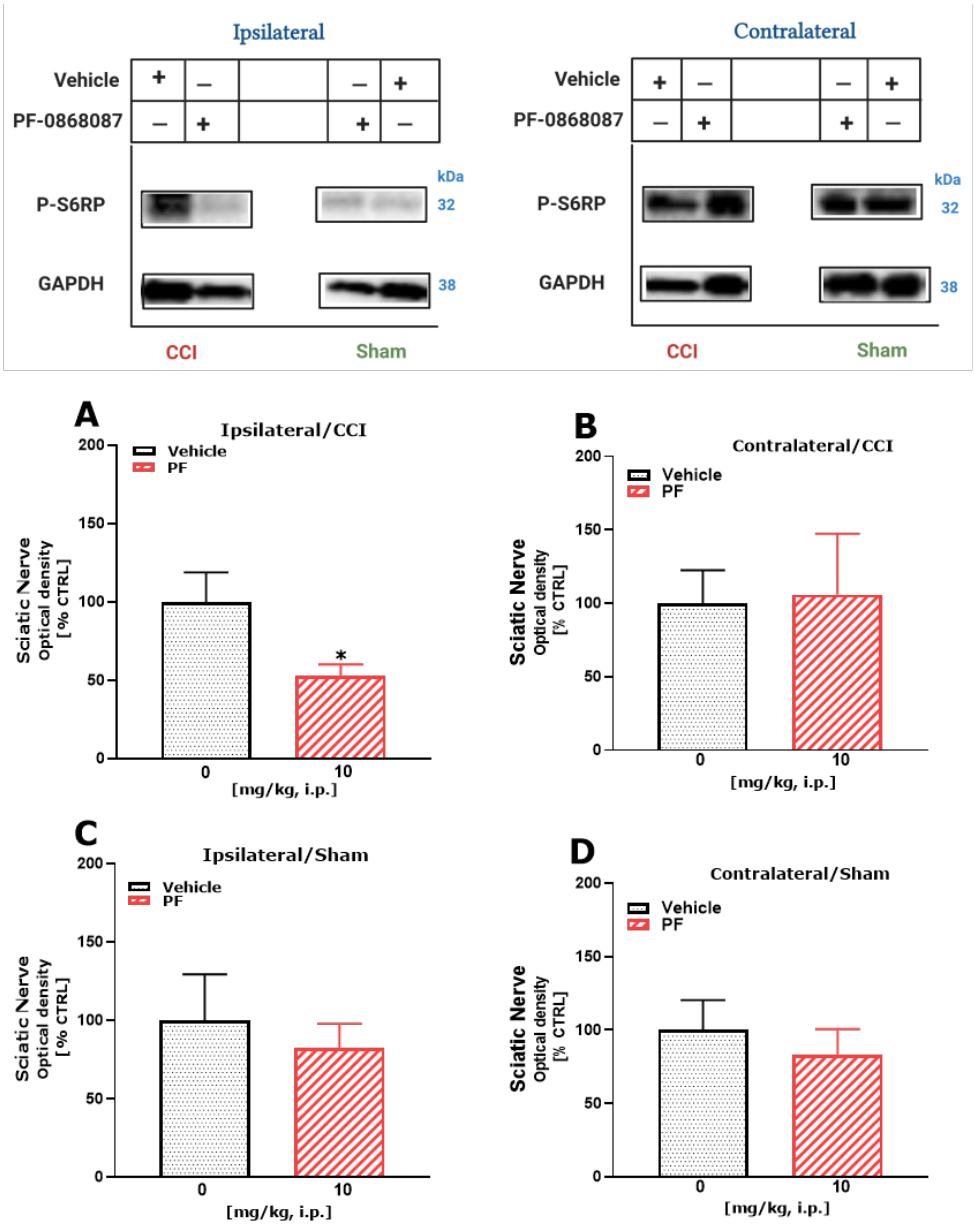


Figure 7.7 Chronic systemic i.p. administration of PF-0868087 decreased the phosphorylation of S6RP in the sciatic nerve in the CCI model of neuropathic pain in mice. (A and B) Effects of i.p. injection of 8680871 (10 mg/kg), or vehicle (V), on the level phospho-S6RP in **(A)** ipsilateral and **(B)** contralateral side of the sciatic nerve. **(C, D)** represent the effect on the sham control mice. Mice received an i.p. injection of PF-0868087 or vehicle 1 dose every 24 hours at same time for 4 constitutive days before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. n=5 to 6 per each experimental group, each graph represents the phosphoprotein mean ± SEM values. The asterisk (*) denotes significance *vs* vehicle controls; *P<0.05 (unpaired Student t-test). Representative Western immunoblots are shown above the graph bar.

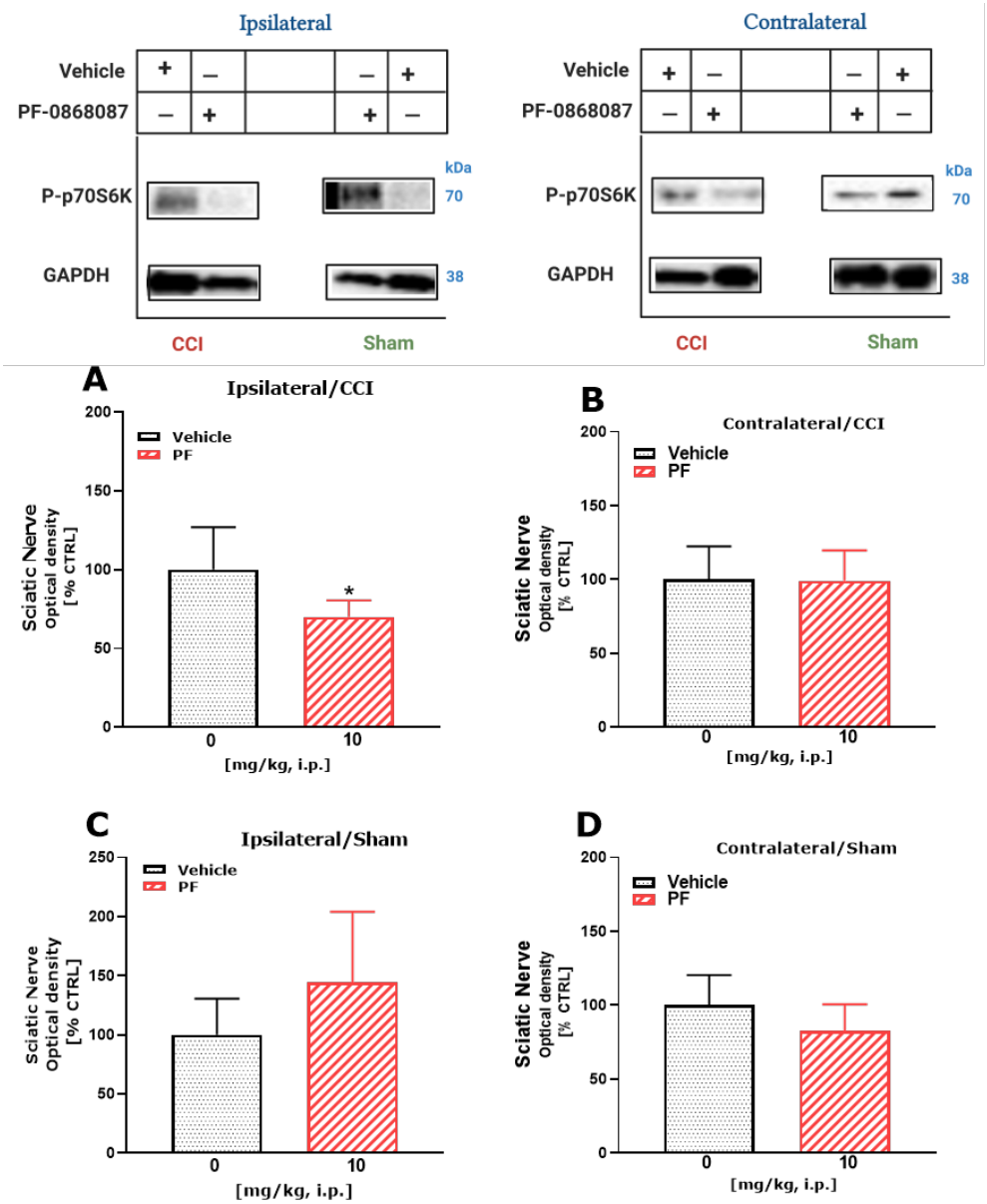


Figure 7.8 Chronic systemic i.p. administration of PF-0868087 decreased the phosphorylation of P70S6k in the sciatic nerve in the CCI model of neuropathic pain in mice. (A and B) Effects of i.p. injection of 8680871 (10 mg/kg), or vehicle (V), on the level phospho-P70S6K in **(A)** ipsilateral and **(B)** contralateral side of the sciatic nerve. **(C, D)** represent the effect on the sham control mice. Mice received an i.p. injection of PF-0868087 or vehicle 1 dose every 24 hours at the same time for 4 constitutive days before sacrifice. The intensity of the bands for each antibody was normalized with the intensity of its appropriate GAPDH signal. n=5 to 6 per experimental group, each graph represents the phosphoprotein mean ± SEM values. The asterisk (*) denotes significance *vs* vehicle controls; *P<0.05 (unpaired Student t-test). Representative Western immunoblots are shown above the graph bar.

7.5 Discussion

For the first time our current research establishes a conclusion that, administration of a CNS-sparing H_3R antagonist PF-0868087 reduced mTORC1 activity as observed by inhibition of its downstream targets, 4E-BPs, S6K, p70S6K in neuropathic pain in mice. The data obtained from western blotting analysis strongly support the behavioural results confirming the hypothesis about the involvement of the mTORC1 pathway in the regulation of antinociceptive efficacy of PF-0868087 as previously proposed by Yan et al., (2014) and supported by our observation in Chapter 5. This data emphasises the significant role of the mTORC1 pathway in the antinociceptive effect of H_3R antagonists that may suggest histamine independent mechanism. The present findings, therefore, shed new light on H_3R and its signalling pathways in the context of neuropathic pain and suggest that H_3R is a potential target in therapy for this type of chronic pain where mTORC1 signaling may play a critical role.

As mentioned earlier, several studies reported that H_3R antagonists showed promising analgesic and anti-inflammatory effects in animal models of pain. *For example,* Hsieh and colleagues (2010) found that systemic administration of the potent and selective H_3R antagonist, GSK189254 produced dose-dependent efficacy in an *in vivo* model of osteoarthritic pain (Hsieh et al., 2010). In 2011, Hough and Rice demonstrated that the inhibition of H_3R activity using inverse agonist and/or antagonist drugs in a variety of acute and chronic pain experiments, lead to a reduction of several types of pain, including neuropathic pain (Hough and Rice, 2011). Similarly, H_3R antagonists have also been reported to be effective in reducing allodynia and hyperalgesia, in *in vivo* models of both neuropathic and inflammatory pain (Hsieh et al., 2010). Also, our work shown in the previous Chapters, demonstrate the antinociceptive effect of PF-0868087, a CNS-sparing histamine H_3R antagonist in the CCI model of neuropathic pain. Although many mechanisms of action have been suggested for the antinociceptive efficacy of H_3R antagonist, the exact underlying mechanism is largely unknown. It has been demonstrated that the H_3Rs are involved in the activation or modulation of several signaling pathways, including the inhibition of the cAMP/PKA pathway, inhibition of the Na+/H+ exchanger, mobilization of intracellular Ca^{2+} stores, activation of mitogen-activated protein kinases, inhibition of

voltage-dependent calcium channels, and activation of G protein-activated inwardly rectifying potassium channels(Leurs et al., 2005; Alexander et al., 2015 ; Nieto-Alamilla et al., 2016 ; Vázquez et al., 2020). In pain, Chen et al., 2017 presently found that H_3R antagonists inhibit the MAPK/PI3K signaling pathways that have been proven to play a critical roles in the development and maintenance of chronic pain (Chen *et al.*, 2017). Also, a number of pre-clinical studies have demonstrated that the activation of the spinal noradrenergic system plays an important role in the efficacy of H_3R antagonists in neuropathic and inflammatory pain (Malmberg-Aiello et al., 1994; Chaumette et al., 2018). Furthermore, other mechanisms have also been proposed to explain the antinociceptive effects attributed to H_3R antagonist involving inhibition of Na^+/H^+ exchanger pathway and inhibition of $cAMP/K^+/Ca^{2+}$ mobilization (Leurs et al. 2005; Bongers et al., 2007) . However, in our work, we hypothesise that H_3R may produce its antinociceptive effects *via* the inhibition of PI3K/Akt/GSK-3β /mTOR pathway as previously suggested by Yan et al., (2014) as an important cellular pathway contributing to peripheral neuropathy (Yuan et al., 2019; Chen et al., 2017).

It has been previously shown that peripheral nerve injury leads to the activation of PI3K and then subsequent inhibition of the GSK-3β axis which could positively regulate axonal elongation that potentially contributed to the mechanism of chronic pain (Chen *et al.*, 2017; Rahmati, Taherabadi and Mehrabi, 2015). Indeed, more recent studies have suggested that histamine *via* H_3R exert part of their actions by controlling the activity of the AKT/GSK3β pathway (Bongers *et al.*, 2007; Mariottini *et al.*, 2009; Bongers, Bakker and Leurs, 2007). Therefore, selective blockade of the H_3R may lead to the activation the GSK-3β and subsequent inhibition of the mTORC1 activity, as shown by reduced phosphorylation of mTORs and its downstream effectors 4E-BPs, S6K and p70S6K (Chiang and Abraham, 2005; Yan *et al.*, 2014; Mariottini *et al.*, 2009).

In the present study, we have found that chronic administration of selective H_3R antagonist, reduced the activity of mTORC1 and its major downstream effectors 4E-BPs, S6K and p70S6K in the dorsal horn of CCI mice as shown by the reduction in the ratio of phospho- mTOR/total and its downstream effectors 4E-BPs, S6K and p70S6K.

Previous investigations on the signaling cascades found that peripheral nerve injury enhanced nociceptive signaling within the dorsal horn of the spinal cord *via* the PI3K/Akt/mTOR signaling pathway and this pathway was successfully inhibited by administration of rapamycin or its analogues that are potent and selective, mTORC1 inhibitors (Géranton et al., 2009; Gregory et al., 2010; Obara et al., 2011), suggesting that neuropathic pain models, which are characterized by the development of secondary hyperalgesia can benefit from treatment with mTORC1 inhibitors (Lisi et al., 2015). Indeed, pre-clinical studies in neuropathic rats demonstrated that peripheral systemic administration of rapamycin significantly attenuated mechanical allodynia (Jiménez-Díaz et al., 2008; Géranton et al., 2009). In agreement with these observations, also Obara et al., (2011), found that i.pl. or i.p injection of mTORC1 inhibitor temsirolimus whether after signal dose (acute administration) or administered for four days (chronic administration) after induction of pain, significantly reduced mechanical and cold allodynia induced by nerve injury in mice. Interestingly, mechanical allodynia was also shown to be reduced when metformin (inhibits mTORC1 pathway by increasing AMPK activation) was administered on a daily basis (Melemedjian et al., 2011). In addition to these observations, targeting of mTORC1 by using mTORC1 inhibitors were also found to have beneficial impacts in different neuropathic pain models such as CCI (Zhang et al., 2013; Marinelli et al., 2014), SNL (Asante et al., 2010; Melemedjian et al., 2011). All these findings indicate that mTORC1 plays a significant role in the regulation of chronic pain and selective targeting of this pathway may represent a potential therapeutic intervention target for chronic pain, particularly in the case of neuropathic pain.

Previous research has suggested that the selective inhibition of the H_3R is associated with the inhibition of mTORC1 activity, and the current research lends support to this theory. In primary cultured neurons, Yan et al (2014) discovered that an H_3R antagonist inhibits the phosphorylation of the mTOR/P70S6K pathway (Yan et al., 2014). Furthermore, Zhao et al (2020) discovered that inhibiting H_3R reduces the phosphorylation of the PI3K/Akt/mTOR signaling pathways in human non-small cell lung cancer cells. Similarly (Zhao et al., 2020). A connection between the H_3R and mTOR pathways has been established as a result of all of these findings. We present

here additional evidence for this connection, as we discovered that targeting H_3R with the administration of the CNS-sparing H_3R antagonist, PF-0868087, significantly reduced the activity of the mTOR pathway.

To further verify the effect of PF-0868087 on mTORC1 and its downstream effectors activity in peripheral neurons, the phosphorylation level of S6K and p70S6K (downstream effectors of mTORC1) in the sciatic nerve was also detected. The result indicates that PF-0868087 reduced the phosphorylation level of both S6K and p70S6K in CCI mice but did not affect sham control mice. Unfortunately, while phospho-S6 and phosphor-p70S6K was observed in the sciatic nerve, mTORC1 phosphorylation was not detected (for more detail see in the explanation of limitation section in the last Chapter). Despite that, while phospho-S6 and phospho-p70S6K levels were detected and these are consequently considered to be a reliable biomarker for the activation/inhibition of mTORC1 pathway (Chiang and Abraham, 2005; Yang et al., 2014; Lisi et al., 2015). Interestingly, our observations are consistent with previous research which linked mTORC1 and its downstream effectors S6K and p70S6K level in the sciatic nerve in pre-clinical neuropathic model. This indicates that the mTORC1 and its downstream pathway may be involved in the antinociceptive effects of PF-0868087.

In conclusion, the present study showed for the first time that H_3R is potentially involved in the regulation of pain perception through a mechanism directly involving inhibition of the mTORC1 pathway (through histamine-independent mechanisms).

GENERAL DISCUSSION AND CONCLUSION

This final Chapter summarises the main findings from the experiments conducted and presented in this thesis. It also aims to address the limitations, implications, and future directions that could help to develop pathways for the more effective treatment and management of acute itch and chronic neuropathic pain, potentially leading to future clinical developments.

8.1 SUMMARY OF FINDINGS AND THEIR IMPLICATIONS

This thesis is specifically focused on the mechanisms within peripheral sensory pathways involved in the regulation of acute itch and chronic neuropathic pain, in particular involving mechanisms based on histamine and histamine receptors' signalling . Results obtained during these studies may provide novel insight into the peripheral mechanism of histamine-dependent itch and chronic neuropathic pain that remains in the clinic the most refractory diseases, which affects millions of people across the globe. Data presented here help to define the involvement of histamine and its receptors in these two conditions. In my opinion, these studies may lead to further assessment of the role of the peripheral histaminergic system in both disease states or other related conditions and may help to develop new and highly selective ligands showing fewer side effects for a wide variety of resistant acute itch and refractory neuropathic pain.

Generally, the relationship between histamine and both conditions has been argued for nearly a century. Numerous research have been published examining histamine's capability to induce and mediate itch and pain and the effect of antihistamines on the treatment and control of symptoms in both sensory processes. However, despite the effort, our understanding of the link between histamine system and these two conditions is rather limited. Therefore, we designed experiments to address this issue as well and used *in vivo* pre-clinical models that are well accepted in the field. In the light of the findings presented in this thesis, our fundamental understanding of histamine

biology has advanced significantly, particularly our growing understanding about the role of new histamine receptors H_3R and H_4R in the regulation of itch and pain. We specifically showed a new utilisation of several new generation antihistaminergic compounds targeting the peripheral histamine system in a novel and interesting way. This includes Votucalis, a novel CNS-sparing highly potent histamine scavenger protein; PF-868087, a selective and peripherally-acting CNS-sparing H_3R antagonist; and VUF16129, a novel light-switchable ligand targeting H_4R. An important aspect of our investigation is related to the use of ligands with a different mechanism of action and restricted penetration to the CNS to minimise CNS-related adverse effects, in contrast to other histamine ligands that display high CNS permeability. Given pharmacological (e.g., high H_3R selectivity of PF-0868087) and chemical (e.g., the high solubility of PF-0868087, the protein structure of Votucalis) profile of these ligands, including PF-0868087's application in a clinical trial on allergic rhinitis, our experiments may lead to an immediate novel avenue for the improvement of neuropathic pain control in humans. In addition, as mentioned above, we investigated the role of peripheral histamine receptors in acute itch using photo-switchable ligands to control pharmacological activity using a specific wavelength of light. For the first time, we used VUF-16129 a photo-switchable compound that can be reversibly switched *via* light between two isomers (*trans-cis*) corresponding to high and low pharmacological activity showing the importance of H_4R in the regulation of histaminergic itch.

8.1.1 Votucalis attenuated histaminergic itch and mechanical hypersensitivity in neuropathic pain

Chapter 3 and 5 demonstrate that Votucalis, a novel CNS-sparing and high-affinity recombinant histamine binding protein, produced both anti-pruritic and anti-nociceptive effects in mouse models of acute itch and chronic neuropathic pain following both systemic and peripheral administrations, which represent commonly used routes of drug applications in clinical practice. The main finding, however, highlights the advantage of localised peripheral administration of Votucalis, as the anti-pruritic and anti-nociceptive effects were achieved at lower doses and produced a longer duration of anti-pruritic and anti-nociceptive effects than systemic administration of this compound. This is therefore the first observation for targeting the

peripheral histamine system by Votucalis to deliver a novel strategy (histamine capture) for potentially safer and more efficacious control of acute itch and chronic neuropathic pain. In fact, *in vivo* neutralisation of locally secreted histamine by locally administered Votucalis has also been shown previously in a wide range of anti-inflammatory effects in mouse models of acute respiratory distress syndrome (ARDS) or allergic asthma (Ryffel et al., 2005). These observations, along with our own, suggest that sequestration of histamine by Votucalis may have therapeutic potential as a non-sedating and non-addictive agent. Our observation may have an important therapeutic impact as current itch management strategies rely primarily on antihistamines, corticosteroids and/or immunosuppressive medications, which are ineffective in the vast majority of cases. In addition, systemic use of these medications for an extended period may increase the risk for developing adverse drug reactions and finally lead to discontinuation of treatment. As a result, our findings may point to a novel therapeutic strategy for the more effective management of itch, particularly in cases where conventional treatments have failed to provide satisfactory results. In addition, Votucalis may also provide a powerful research tool that may generate new insights into the understanding of the role of histamine in a range of pathological conditions where this amine plays an important regulatory function. Figure 8.1 shows a schematic illustration of possible mechanisms by which Votucalis affects itch and neuropathic pain.

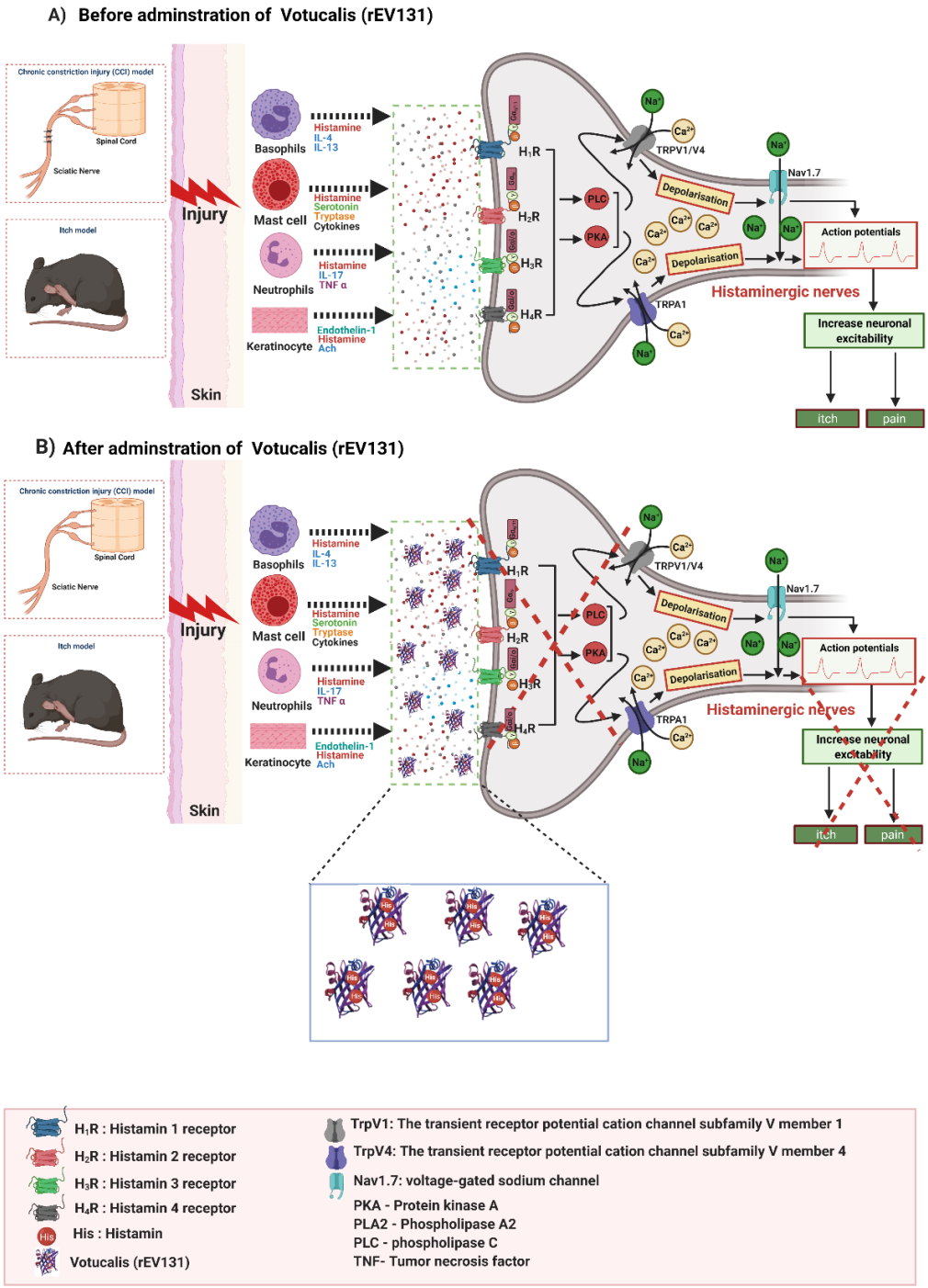


Figure 8.1 Schematic illustration of possible mechanisms by which Votucalis affects itch and neuropathic pain. Votucalis attenuated peripheral neuropathic pain condition and acute itch by binding endogenous histamine at two internalized sites; one of a very high affinity and this allows to prevent histamine from binding to HRs, resulting in a decrease of the neuronal excitability and subsequently may reduce pain and itch sensations. The information in the figure is partially based on Leslie, 2013; Green and Dong, 2016; Meixiong and Dong, 2017; Dong and Dong, 2018.

8.1.2 Votucalis allowed unravelling the role of H_1R, H_2R and H_4R in acute itch

Chapter 3 demonstrated that H_1R and H_2R as well as H_4R play an important role in the regulation of itch sensation. The studies in this Chapter used selective histamine receptors antagonists and different routes of their administration to distinguish the role of peripheral and central HRs in the control of itch. Our study further indicates and confirms that histaminergic itch is predominantly mediated by peripheral H_1R and H_2R, and less by central H_1R and H_2R, as peripherally administered selective antagonists of these receptors inhibited histaminergic itch at doses that were either not or less effective with systemic route of administration.

These findings provide new insight into the mechanism of H_1R mediated anti-itch effect as some studies have argued that the anti-itch effect resulting from H_1R antagonism is due to sedation, rather than the direct blockade of H_1R on sensory neurons (Bell et al., 2004; Imaizumi et al., 2003; Monczor and Fernandez, 2016; Krause and Shuster, 1983). In line with our observations, both mRNA and functional expression of H_1R was shown on peripheral neurons displaying characteristics of C-fibers that are potentially involved in the regulation of itch (Kashiba et al., 2001; Rossbach et al., 2011). In addition, Bell et al. (2004) found that the intradermal administration of a H_1R agonist caused dose-dependent scratching in mice (Bell et al., 2004) showing functional involvement of this HR in the itch activation. Based on the present results, we have provided data to support another previous hypothesis that peripheral, rtaher than central, H_1R is critical for *the mediation of histamine*-induced itch. However, it should be noted that clinically available H_1R antagonists failed to completely reduce the scratching behaviour in some types of itch, therefore, suggesting that also other HRs are potentially involved and critical in the regulation of histaminergic itch.

Interestingly however, the role of H_2R in itch is still somewhat controversial. In this thesis our findings also extended the understanding of the role of peripheral H_2R in itch as the available literature is rather inconclusive and indeed controversial (Bell et al., 2004; Rossbach et al., 2011). It seems that H_2R-mediated anti-itch response is due to antagonism of peripherally expressed H_2R that are known to be present on primary afferent neurons (Kajihara et al., 2010) although their functional expression has not yet

been confirmed (Rossbach et al., 2011). The proposed role of peripheral H_1R and H_2R in itch transmission can therefore be further emphasised by the use of Votucalis as its anti-itch effects was stronger by antagonism specifically targeting peripheral H_1R and H_2R.

In addition, in line with others, we also found that antagonism at H_4R suppressed histaminergic itch (Dunford et al., 2007; Rossbach et al., 2011). This effect was however observed after both peripheral and systemic administration of a selective H_4R antagonist and in this experimental setting, we were not able to distinguish whether peripheral or central H_4R may play a more crucial role. Studies using Votucalis and VUF16129 shed more light on the insight of the role of this HR in the regulation of itch and it seems as both peripheral and central H_4R may be important depending on the way of targeting this HR. Figure 8.2 summarises possible mechanisms by which HR antagonists used in this study (mepyramine, ranitidine and JNJ7777120) affect histaminenegic itch.

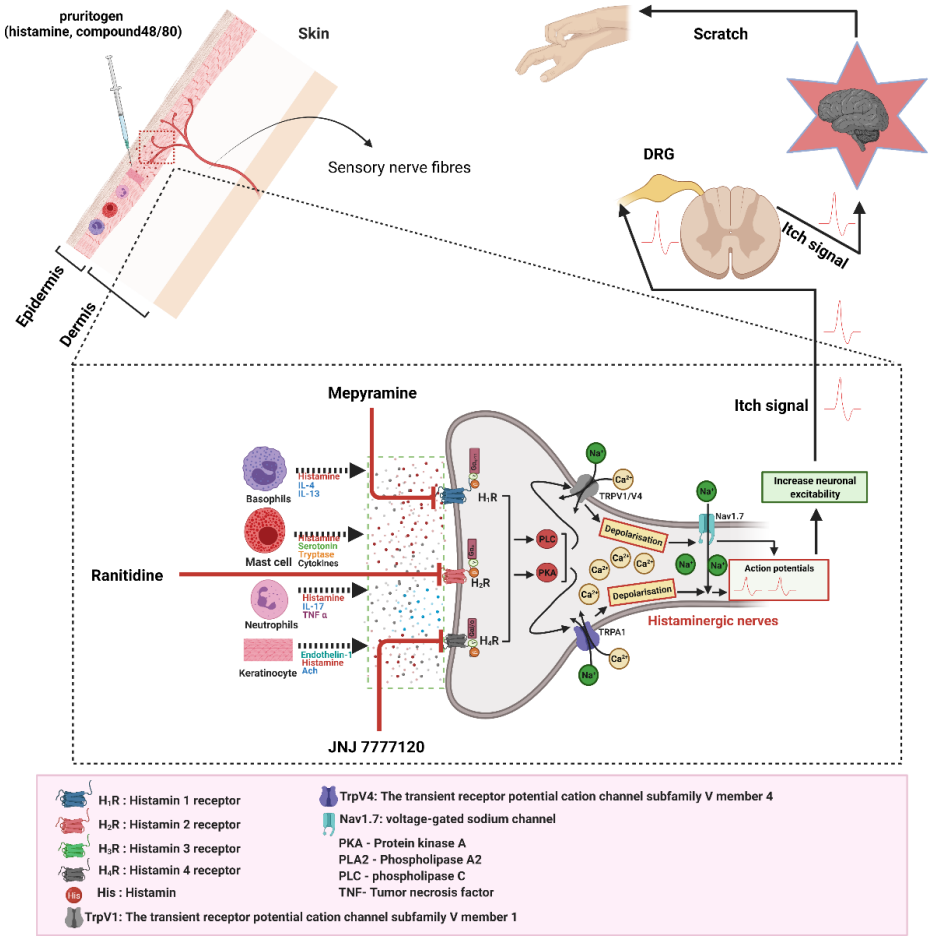


Figure 8.2 Schematic illustration of possible mechanisms by which mepyramine, ranitidine and JNJ7777120 affect itch. Information in the Figure is partially based on Leslie, 2013; Green and Dong, 2016; Meixiong and Dong, 2017; Dong and Dong, 2018.

An interesting finding from this research about the regulation of itch sensation comes from experiments where Votucalis was used in the combination with other selective HR antagonists. This approach produced a consistent and significant inhibition of itch when compared with the anti-itch effects produced by each drug alone either it is Votucalis or HR antagonists. Specifically, the anti-pruritic effects of Votucalis became more significant (produced stronger anti-itch effect) when co-administered with locally peripherally administered H_1R and H_2R antagonists and, on the other hand, only systemically administered H_4R antagonist. In other words, Votucalis provided inhibition of histaminergic itch around 62 %, which was further increased by both peripheral H_1R and H_2R or central H_4R antagonism up to around 85, 90 and 81 %, respectively (peripheral H_1R and H_2R or central H_4R antagonism without Votucails inhibited histaminergic itch around 64, 53 and 39 %, respectively). These observations further confirmed the involvement of peripheral H_1R and H_2R in the regulation of acute itch as well as to some extend showed the predominant involvement of central H_4R although this could be potentially linked to the nature of the pharmacological activity of Votucalis.

In conclusion, this part of the thesis is adding further insight on the involvement of H_1R, H_2R, and H_4R in acute itch based on *in vivo* research. Stimulation of these receptor subtypes seems to be involved in the facilitation of histamine-induced itch, and selective inhibition of H_1R, H_2R, and H_4R inhibited histamine-induced itch, *via* peripheral and/or central mechanisms. Findings suggest a specific and predominant involvement of peripheral H_1R, H_2R and central H_4R in the regulation of acute histaminergic itch.

8.1.3 VUF16129 allowed unravelling the role of H_4R in acute itch

In Chapter 4, the studies on H_4R provided a new path for the exploration of the physiological role of histamine and H_4R in acute itch, as well as indicated for a new drug target for the development of novel antihistamines. Numerous previous studies aimed at the identification of selective H_4R antagonists to help unravel the pharmacology of H_4R relative to other HRs. The discovery of the selective H_4R antagonist JNJ 7777120 was vital for showing a role for H_4R in inflammation and pruritus. While this compound has been very successful as a tool for understanding the function of the HR, it has drawbacks,

including a short *in vivo* half-life and hypoadrenocorticism toxicity in rats and dogs, that prevented advancing it into clinical studies. Further research led to the discovery of JNJ 39758979, which, similar to JNJ 7777120, was a potent and selective H_4R antagonist and showed anti-inflammatory and anti-pruritic activity pre-clinically. JNJ 39758979 advanced into human clinical studies and showed efficacy in reducing experimental pruritus and in patients with atopic dermatitis. However, the development of this compound was terminated due to the occurrence of drug-induced agranulocytosis.

It is necessary to use highly selective and potent ligand agonists as pharmacological tools to investigate the involvement of receptors in physiological and pathological conditions, both in pre-clinical and clinical studies, in order to better understand how receptors function. Many H_4R agonists that were discovered and used in pre-clinical models, on the other hand, were found to have only weak or insufficient H_4R selectivity when tested in humans (Igel et al., 2010; Acton, 2013). The evaluation of H_4R agonists in animal models is further complicated by differences between human and rodent receptors in terms of affinity, potency, and selectivity (Igel et al., 2010; Acton, 2013). As a result, further research into the detailed function of a new selective H_4R ligand is required. Photo-pharmacological regulation of drug activities for their intended targets is gaining popularity as a promising method for improving pharmacological activities. It is possible to employ specific ligands and modulate their activity under the influence of a specific wavelength of light to optically control the activity of a specific type of receptor using this approach, which is described in detail in Chapter 4 (Lerch et al., 2016; Zeng et al., 2021). When exposed to light, the photo-switching ligand may absorb a photon, resulting in a photo-chemical reaction that causes structural rearrangements in the ligands that come into contact with their receptor. These structural rearrangements can have an impact on the biological activity of the ligands that come into contact with their receptor (Ricart-Ortega et al., 2020).

This study is the first to demonstrate *in vivo* the role of H_4R in acute itch by employing a photo-switchable agonist able to modulate its pharmacological activity at H_4R in mice. Specifically, after being exposed to UV light for a short period of time, *trans*-VUF16129 can photo-switch to another isomer (*cis*), which differs in their affinity, potency, and

efficacy toward H_4R. Itching behaviour associated with activation of H_4R was observed with *trans*-VUF16129 when it was injected locally into the nape of the mouse neck. This behaviour was not observed with cis- VUF16129 upon manipulation of the drug activity with a specific light. In our study, photo-pharmacological approaches have been used to obtain the first *in vivo* evidence to suggest that also peripheral H_4R, in addition to our observation regarding central H_4R, is important in the regulation of itch. In summary, developing biological data using a variety of methods that target H_4R has the potential to improve our understanding of pathophysiology and sensory processing mechanisms, ultimately leading to the development of potential new drugs for the treatment of itching.

8.1.4 PF-0868087 reduced mechanical hypersensitivity in neuropathic pain

In Chapters 6 and 7 we used a combination of *in vivo* and *biochemical* approaches to further confirm the important role of H_3R in the regulation of neuropathic pain. We first showed the antinociceptive effects produced by the selective and peripherally acting / CNS-sparing H_3R antagonist PF-0868087 in a mice model of neuropathic pain. The important finding from this study indicates the predominant involvement of peripheral H_3R in the regulation of mechanical hypersensitivity neuropathic pain. In addition, using immunoblotting approaches, we determined the involvement of neuronal mechanisms in the regulation of H_3R mediated analgesia and showed this to be regulated *via* mTORC1 pathway. Specifically, we showed that administration of PF-0868087 associated with the inhibition of peripheral H_3R attributed to the inhibition of mTORC1 activity in the spinal cord as PF-0868087 decreased the phosphorylation levels of mTORC1 and its downstream targets including P-p70 S6 kinase and P-S6 ribosomal protein. It is highly possible that this mechanism directly underlies the analgesic effect achieved after administration of PF-0868087 acting *via* peripheral H3R in neuropathic pain.

From the time when the cloning of H_3R in 1999 was performed by Lovenberg and co-workers, there has been an extended interest within the pharmaceutical industry to find and develop antagonist/inverse agonist ligands to this HR, outlined by several companies creating around 100 obvious applications for H_3R ligands. The obvious writing has proposed a few diseases for which the enmity of H_3R may offer treatment.

Consequently, selective targeting of H_3R by different ligands showed potential therapeutic effects in pre-clinical animal models of obesity (Hancock, 2003), diabetes (Yoshimoto et al., 2006), seizures (Kakinoki et al., 1998), neuropathic pain (Medhurst et al., 2008; Hsieh et al., 2010), multiple sclerosis (Krementsov et al., 2013), cerebral ischaemic injury (Yan et al., 2014), sleep-wakefulness (Hass et al. 2008) and several cognitive diseases, such as Alzheimer's disease, Parkinson's disease and attention deficit hyperactivity (Passani et al. 2004; Jope and Johnson, 2004; Leurs et al. 2005; Bongers et al., 2007).

A search for new H_3R selective ligands also involved a design that would allow to reduced CNS-mediated effects, particularly in conditions where such effects are considered as unwanted e.g., allergic rhinitis. Such an approach can be considered also as desired in conditions that require regulation of the sensory system e.g., chronic pain. Anatomical localisation of H_3R may support this approach as H_3R appears to be expressed at both histaminergic and nonhistaminergic neurons. On the histaminergic neurons, presynaptic H_3R is expressed as autoreceptors that control histamine release via negative feedback mechanism (Arrang et al., 1983; Morisset et al., 2000). In addition, H3R is expressed as heteroreceptors on the non-histaminergic neurons that control the release of many other neurotransmitters such as Ach, dopamine, noradrenaline, 5 -HT , and a variety of neuropeptides in both CNS and PNS (Clapham and Kilpatrick, 1992; Schlicker et al., 1994; Yan et al., 2014; Blandina et al., 2010; Hsieh et al., 2010; Obara et al., 2020). It has been widely proven that H_3R are more distributed in CNS, but also, H_3R mRNA is located in various non-brain tissues, such as stomach, intestines, adipose tissue, heart, lung, dorsal root ganglia, superior cervical ganglia, skin and, importantly, on specific types of primary sensory neurons Aβ fibers, Aδ fibers terminating on deep dermal blood vessels (Cannon et al., 2007; Obara et al., 2020; Heron et al., 2001; Hough and Rice, 2011).

Our data suggest that it may be possible to effectively control mechanical hypersensitivity in neuropathic pain caused by nerve lesions using a selective H_3R antagonist PF-0868087 that specifically acts at the peripheral level blocking the activity of peripheral H_3R. Interestingly however, the role of H_3R, particularly peripheral H_3R in neuropathic pain has not been fully explained, and most of the results in this area are remain somewhat controversial. The potential involvement of H_3R in pain processing

has been indicated by previous studies using H_3R agonists and antagonists in different types of pain models (Hsieh et al., 2010; Hough and Rice, 2011). Hsieh and colleagues (2010) showed that systemic administration of the potent and selective H_3R antagonist; GSK189254, produced dose-dependent efficacy, in an *in vivo* model of osteoarthritic pain (Hsieh et al., 2010). In 2011, Hough and Rice demonstrated that inhibition of H_3R activity using inverse agonist and/or antagonist drugs in a variety of acute and chronic pain experiments, lead to a reduction of several types of pain, including neuropathic pain (Hough and Rice, 2011). H_3R antagonists have also been reported to be an effective treatment in reducing allodynia and hyperalgesia, in *in vivo* models of both neuropathic and inflammatory pain (Hsieh et al., 2010). In contrast with this hypothesis, some studies suggest that H_3R agonists have anti-inflammatory as well as some antinociceptive properties (Rouleau et al., 1997; Cannon et al., 2003). Furthermore, data gathered from the literature suggested that antagonism of H_3R protects against neurotoxicity caused by N-Methyl-D-aspartate (NMDA) in *in vitro* cortical neurons (Dai et al., 2007).

We are the first to demonstrate that the antinociceptive effect of PF-0868087 is due to its activity on peripheral H_3R in neuropathic pain. Given the pharmacological (high H_3R selectivity of PF-0868087) and chemical (high solubility of PF-0868087) profile of this compound, including it completed toxicology studies and now read to enter the first phase of clinical trials for allergic rhinitis (Lunn et al., 2012), our experiments may lead to an immediate novel avenue for the improvement of mechanical hypersensitivity associated with neuropathic pain in humans.

8.1.5 PF-0868087 allowed unravelling the interaction between H_3R and mTORC1 pathway

This thesis shows for the first time a potential interaction between H_3R and mTORC1 pathway as a mechanism underlying an effective pain control resulting from the inhibition of peripheral H_3R that has been targetted by a selective and peripherally acting/CNS-sparing ligand.

Many hypotheses explained the mechanism by which H_3R antagonist induce their antinociceptive effects. It was shown to involve the activation of the noradrenergic fibers (McGaraughty et al., 2012) or by controlling the release of different

neurotransmitters via heteroreceptors H_3R negative feedback mechanism (since blocking of H_3R is known to increase the release of ACh, dopamine, noradrenaline, serotonin and substance P in the CNS (Gemkow et al., 2009; Blandina et al., 2010; Giannoni et al., 2010; Obara et al., 2020). Another hypothesis explained this antinociceptive effect of H_3R antagonist via the TRPM8 receptor, a member of the TRP channel family (Knowlton et al., 2013; Obara et al., 2020) as well as the inhibition of Na^+/H^+ exchanger pathway and the inhibition of K^+ /Ca^{2+} mobilization pathway (Leurs et al. 2005; Bongers et al., 2007).

In this thesis, we examined an alternative strategy where H_3R antagonist could act *via* histamine independent pathway. Interestingly, Yan et al. (2014) showed that blockade of H_3R by selective antagonists (E-162) significantly activated GSK-3β, a downstream kinase of H_3R that lead to the inhibition of themTORC1 signalling pathway (Yan et al., 2014). Based on previous work it was indicated that activation of mTORC1 results in phosphorylation and activation of its downstream effectors 4EBP1 and p70S6K leading to the initiation of mRNA translation and therefore to the synthesis of new proteins involved in peripheral and central neuronal plasticity (Switon et al., 2017; Jefferies et al., 1997). Currently, neuronal plasticity that is associated with structural and functional changes in neurons (Kuner and Flor, 2017; Bak et al., 2021), is a widely accepted feature of several types of chronic pain and in particular neuropathic pain (Bak et al., 2021; Asante et al., 2010). The activity of mTORC1 is modified in a wide range of pathological states, such as cancer, pain and neurodegenerative disorders (Jaworski and Sheng, 2006; Asante et al., 2010; Switon et al., 2017; Obara et al., 2011). Indeed, previous investigations on the mTORC1 signalling cascade found that peripheral nerve injury enhanced nociceptive signaling within the dorsal horn of the spinal cord via the PI3K/Akt/mTOR signalling pathway and this pathway was successfully inhibited by administration of mTORC1 inhibitor such as temsirolimus (Géranton et al., 2009; Gregory et al., 2010; Obara et al., 2011). Specifically, administration of mTORC1 inhibitor, rapamycin or its analogues, which blocks the phosphorylation of mTORC1 and its downstream effectors, has been shown to alleviate mechanical hypersensitivity associated with nerve damage in neuropathic pain models (Gregory et al., 2010; Zhang et al., 2013; Obara et al., 2011). In line with these observations and the work of Yan et

al. (2014), it may be possible that the effects produced by PF-0868087 may be associated with the direct interaction between H_3R and mTOC1 pathway. This may be supported by the observation that chronic administration of PF-0868087, a selective H_3R antagonist, reduced activity of mTORC1 and its major downstream effectors in the dorsal horn in neuropathic mice. Thus, it may seem that mTORC1 pathway may represent a novel molecular mechanism for pain relief following the application of the H_3R antagonist.

8.1.6 Advantage of peripheral administration over systemic drug delivery

Despite advances in the basic and clinical understanding of the etiology and diagnosis of acute itch and neuropathic pain, the current state of available treatment for acute itch and chronic neuropathic pain remains inadequate. To date, no medication has demonstrated tolerability and long-term efficacy in the treatment of neuropathic pain, and even with existing medications, less than half of patients achieved pain control (Ciaramitaro et al., 2019; Saat, 2020). In addition, patients are exposed to the potential side effects and drug interactions associated with current medications, particularly with systemic treatment options (e.g., Chong and Bajwa, 2003; Haanpää et al., 2010; Bates et al., 2019; Dickenson and Patel, 2020).

In contrast to systemic therapies, localised peripheral targeting agents that act on the PNS are effective in providing rapid and targeted itch and pain relief without the side effects associated with systemic therapies (Sommer and Cruccu, 2017). Recently, Food and Drug Administration (FDA) approved two local application drugs for the treatment of neuropathic pain (capsaicin 8% patch and lidocaine 5% patch). According to the last updated guideline for pain management by the International Association for the Study of Pain (IASP) they recommend the topical capsaicin 8% patch and lidocaine 5% patch as a 2nd line therapy for neuropathic pain. These topical agents may be beneficial in a variety of patient groups because of their demonstrated efficacy, local action, and favourable safety profile with low systemic side effects. In the line with this our tested drugs Votucalis and PF-0868087 have a stronger effect and longer duration with very low concentration compared to the systemic administration. These results are extremely promising for a group of patients suffering from itch and neuropathic pain especially since these drugs were used in pre-clinical and clinical studies and show a favourable safety profile. All these advantages made Votucalis and PF-0868087 a

promising drug for use in clinical trials shortly, however further pre-clinical animal studies are needed before their use in humans.

8.2 METHODOLOGICAL CONSIDERATIONS, CHALLENGES AND LIMITATIONS

As with any PhD research, this study has several limitations associated with the models, time, resources, and impact of the lockdowns due to the COVID-19 pandemic. All these factors affected the research to some degree and should be considered here. Overall, the presented here studies produced useful results and outcomes that could be explored further to advance our knowledge and understanding of processes associated with acute itch and chronic neuropathic pain.

8.2.1 *In vivo* models

Acute itch model. The C57BL/6J mice are one of the oldest and most widely used strains in preclinical biomedical studies, and almost half of citing articles published on PubMed document the use of C57BL/6J (B6/J) strain (Alsubaiyel, 2019; Bryant, 2011). In itch-related studies, C57BL/6J mice are widely and commonly used (Wheeler et al., 2020). In these studies, the selection of the C57BL/6J mice in itch research is due to a variety of reasons. Firstly, C57BL/6J mice were recognised as a standard laboratory strain that had been used in itch research previously presented in many published works (Dunford et al., 2007; Liu et al., 2019; Obara et al., 2015; Shim et al., 2007; Sun and Chen, 2007). Also, C57BL/6J mouse was available and relatively inexpensive. In addition, the B6 mice are physically active, capable of learning a variety of tasks and breed frequently. Moreover, this type of mice was known to have the ability to scratch after administration of pruritogen (Bryant, 2011; Sargent et al., 2016). Previous studies in the field of itch research demonstrated that there are no sex differences in histamine-induced scratching response in mice (Bell, 2004; Wheeler et al., 2020). Despite that to avoid the confounding effects of the oestrus cycle on itch, and to align the study with other studies conducted by our group, only male animals were used despite evidence showing that sex hormones affect mast cell behaviour, itch sensation (Zierau et al., 2012). Also, this thesis was examining whether the protocol for inducing scratching was suitable as a method for studying itch. Scratching in mice was not evoked by any of the experimental control procedures used for example mice did not show any scratch behaviour during the habituation and handling period. Also, mice did not show any

scratch after shaving of the back. The small scratching response was observed after injection of saline or DMSO as vehicle solution used in the experiment, but these responses were insignificant when compared to responses generated by the pruritogen such as histamine, compound 48/80 as described. This part of the experiment was particularly important to confirm that the scratch behaviour was due to administered of pruritogen and not due to the model itself. In this model, human observation and calculating scratching using manual naked-eye observation in mice remain as the gold standard way, however, this manual scoring of videos approach has numerous drawbacks and limitations include extremely time-consuming, tedious, and subject to human error (Brash et al., 2005; Elliott et al., 2017). To avoid the need for manual scratch measurement, it is essential to continue working toward automatic monitoring and quantification of scratching. Furthermore, automated detection will likely give an objective, rapid and efficient measure of itching that will prove to be a useful tool for exploring physiological processes involved in acute and chronic itch, as well as for drug screening of possible anti-pruritic properties shortly.

Neuropathic pain model. To model peripheral neuropathic pain, we used the Bennett and Xie (1988) model of chronic constriction injury (CCI) of the sciatic nerve with slight modifications for mice as described by Obara I et al. (2003, 2013) and Osikowicz et al, (2008). In this model, the CCI injury was produced by tying three loose ligatures (4.0 silk) around the exposed sciatic nerve. The ligatures were tied with a 1 mm spacing, and until a brief twitch in the respective hindlimb is elicited. Consistent with previous studies (Bennett and Xie, 1988; Obara *et al.*, 2013; Obara *et al.*, 2003; Üçeyler *et al.*, 2011; Su *et al.*, 2021), the animals subsequently developed mechanical and thermal sensitivity. Also, in the case of the sham-operated control mice (placebo surgery), The sham-operated control mice underwent surgical procedures identical to that in the CCI group, but the sciatic nerve was isolated and exposed without ligation. Chronic constriction injury is one of the most widely used experimental models of neuropathic pain, can trigger a hypersensitivity reaction in rodents mimic clinical scenarios of neuropathic pain in humans (Honoré *et al.*, 2011; Taylor and Finn, 2014; Berge, 2011).

8.2.2 The impact of COVID-19 and public lockdown

Due to the restrictions imposed by the lockdowns due to the COVID-19 pandemic,

several studies intended for contribution to this thesis were not completed. COVID-19 has had far-reaching implications for academic research that have never before been seen. Libraries have been closed, field sites have been closed, productivity has been reduced, morale has been low, priorities have been rearranged, and entire research projects have been called into question. These changes had a particular impact on my research and any PhD researcher during this period. There are few studies that intended to conduct and include in this thesis, but these could not be completed.

I was planning to carry out *in vitro studies* to investigate the link between H_3R and mTOR following *in vivo* pharmacological changes, we did the surgery for CCI and sham and treated the animals with selected dose, prepared the tissue for immunohistochemistry and unfortunately, due to the lockdown, we stop our experiments, but these studies are now considered in the future directions.

We also planned to start an electrophysiology study on the primary culture of sensory neurons from mice dorsal root ganglia (DRGs). I managed to complete training to isolate the DRG neuron and *culture* for whole-cell patch-clamp electrophysiological recording. However, I was unable to complete all the experiments as planned due to the restrictions and impact of COVID-19 but, again, these studies are now considered in the future directions.

8.2.3 Biochemical analysis

It might be argued that in the western blot experiments presented in Chapter 7, H_3R antagonist (PF-0868087) failed to induce a significant reduction in the protein levels of P- mTOR, mTOR, P-4EBP and 4EBP in the sciatic nerve. However, I showed clearly that the H_3R antagonists suppressed two major markers that reflected mTORC1 activity, P-p706SK and P-S6RP in the spinal cord. The lack of changes in the western blotting technique regarding P-mTORC1, mTORC1, P-4EBP and 4EBP in both sham mice as well in the CCI mice, could be related to the timing when the tissue was collected, or it might necessitate a stronger immune component (antibodies) to give clear bands or the low concentration of the extracted proteins. In addition to this, in our lab we don't have a tissue homogenizer for that reason we planned to homogenize our sample at the Centre for Life at Newcastle, and this required about 45 minutes walk (one-way) this might have

caused even more time constraints and might have affected the protein concentration in the samples during transportations. Moreover, due to the COVID-19 crisis which cause the lockdown of all universities and labs, we kept our homogenized sample in -80^{o}C for about 5 months and then we did the last part of western blot (in sciatic nerve). This long-time gap might be one of the reasons which caused unusual or unexpected bands due to protease degradation, which produces bands at unexpected positions or sometimes not clear band, and this happened in some of our samples. Also, it is important to note that one of the limitation of this study is that in chapter 7 the relation between the peripheral action of PF0868087 and central (spinal cord) modulation of the mTOR pathway is not clear. As previously described and according to several studies mTOR and its downstream effectors play a role in the development of different type of chronic pain such as neuropathic, inflammatory, and cancer (Gregory et al., 2010 ; Shih et al., 2012 ; Liang et al., 2013). Obara et al., 2011 found that systemic i.p. injection of CCI-779 blocks mTORC1 activity in the spinal cord and dorsal roots (Obara et al., 2001). It has been demonstrated that peripheral inflammation increases the activation of the mTOR and its downstream pathway in the dorsal horn of the spinal cord (Lisi et al., 2015). Local administration *via* intraplanar injection of capsaicin significantly increased the phosphorylation level of the S6 ribosomal protein in the dorsal horn neurons, and this activation was abolished by intrathecal administration of rapamycin (mTOR inhibitors) (Géranton et al., 2009 ; Lisi et al., 2015). Also, Gregory et al., 2010 found that peripheral inflammation after i.pl. injection of carrageenan increased the level of S6 phosphorylation in the ipsilateral spinal cord dorsal horn(Gregory et al., 2010).Moreover, DRG and spinal cord dorsal horn mTOR phosphorylation were significantly increased in response to peripheral inflammation caused by an i.pl. injection of complete Freund's adjuvant (Liang et al., 2013). In addition, in a CCI mouse model of neuropathic pain, increased mTOR activation was found in the spinal cord, which was significantly reduced by intrathecal rapamycin administration (Zhang et al., 2013). Finally, local, and intrathecal injection of Rapamycin reduced phosphorylation of mTORC1 downstream targets in peripheral nerve fibers) and spinal cord neurons (Jiménez-Díaz et al., 2008; Géranton et al., 2009; Lisi et al., 2015). All these data suggest that mTOR and its downstream targets in spinal cord are affected by localized peripheral inflammation and inhibition of this peripheral effect by H_3R antagonist may cause

reduction in the mTOR and its downstream targets in the spinal cord. Despite of that, there is still a lack of clear evidence to describe this relation between peripheral action of PF0868087 and central (spinal cord) modulation of the mTOR pathway. For those reasons, future research in this area needs to focus further on the relation between the peripheral HRs and mTOR pathway by using immunohistochemical, western blot and optical imaging analyses in order to provide clear evidence for the link between histaminergic system and mTOR signaling pathway.

8.2.4 Potential systemic effect of subcutaneous administration:

In this study we used subcutaneous administration to represent localised peripheral effect. It is well known that substances administered subcutaneously often are absorbed at a slower rate compared with other parenteral routes, providing a sustained effect (Turner et al., 2011). In the literature, subcutaneous absorption of therapeutics compound is relatively slow and mostly incomplete (Richter and Jacobsen, 2014). Many factors can affect the absorption of a compound from s.c. site to circulation and these include physiochemical properties of substance, volume of administration, site of delivery, pH of the substance, pharmacokinetic profile and molecular weight (Turner et al., 2011 ; Mathaes et al., 2016; Gradel et al., 2018). According to several studies, proteins with molecular weight lower than 16 kDa can be transported from the injection site to the systemic circulation (McDonald et al., 2010 ; Collins et al., 2017 ; Jarvi and Balu-Iyer, 2021); however, proteins with molecular weight greater than 16 kDa, such as Votucalis (23.3 kDa), the s.c. capillaries are practically impermeable (Datta-Mannan et al., 2020). Nevertheless, we cannot avoid the fact that s.c administration may involve an element of systemic effect. Even though the study had some limitations that will affect the scope of the findings, none of these limitations pose a threat to the validity of the findings. Importantly, some interesting observations were made regarding the administration of PF0868087 and Votucalis, both systemically and locally. The comparison of the two methods provided new insights into where these compounds can exert its effects in the pain and itch pathways.

8.2.5 Action of histamine and histamine receptors in non-neuronal cell

In this study, I discussed the role of the histamine and HRs in primary afferent neurons. One of the limitations of this study is that I did not mention the action of HRs on non-neuronal cells such as mast cells in the periphery. I focused here more at the neuronal level not on other signaling pathways that contribute to pain and itch. We cannot avoid the fact that other pathways may play a role in the effect of our drugs. Therefore, there is an essential requirement for future research in this filed to examine the effect of PF0868087 and Votucalis on the mast cell and other non-neuronal cell and this may help to progress towards novel therapeutics and treatments.

8.3 FUTURE STUDIES AND DIRECTIONS

A considerable amount of evidence identified that the histamine system plays an important role in itch and chronic pain in particular neuropathic pain. This role was also recognised in my results obtained from pre-clinical studies. For that reason, this thesis supports the important role of the histaminergic system in regulating acute itch and pain sensitivity and selective targeting of peripheral histamine by using novel substances would result in both anti-pruritic and anti-nociceptive effects that may suggest a new and safe therapeutic approach in the regulation and control of these conditions. Accordingly, it will be interesting to continue some pre-clinical and clinical studies to extend and confirm the impact of these current observations. I present the main future work below.

8.4.1 Future Pre-clinical studies

Study 1: Does peripheral H_3Rs is a therapeutic target for neuropathic pain?

Histamine and histamine receptors ligands are known to produce CNS-mediated adverse effects. The uniqueness of PF-0868087 and Votucalis lies in its chemical and morphological structure that allows for restricted penetration to the CNS to minimise CNS-related adverse effects (Lunn et al., 2012). To completely establish the potential for PF-086087 and Votucalis to improve neuropathic pain control through peripheral H_3R, it is critical to establish behavioural CNS-mediated effect of both drugs. Therefore, in the future, I am aiming to determine the level of motor activity (sedation) and anxiety (latency and entries onto the slopes or platform depending on configuration) in a single

test setting in a continuum using animal models subjected to well-established 3D elevated platform test as previously described by (Ennaceur et al., 2017). A first generation sedative H_1R antagonist will be employed.

Study 2: Do changes in the H_3R activity and signaling underlie the effects of PF-0868087 on neuropathic pain?

The mechanisms involved in H_3R-mediated analgesia in chronic pain are not well identified. To completely establish the involvement of H_3Rs in neuropathic pain and the potential for PF-0868087 to improve pain control, an important part of the proposed research will define the extent to which changes in the H_3Rs activity and signaling underlie analgesic effects of PF-0868087 on neuropathic pain. H_3R has been shown to have multiple downstream effectors that are also known to be involved in the regulation of neuropathic pain e.g. activating mitogen-activated protein kinases (MAPKs) and the gamma isotype of protein kinase C (PKCγ) (Miletic et al., 2015 ; Sanna et al., 2015). While neuropathic pain is associated with phosphorylation of GluA1 in the phosphorylation at Ser831 by PKCγ we hypothesise that the underlying mechanism for PF-0868087 analgesia, particularly long-term effect, involves PKCγ mediated GluA1 Ser 831 dephosphorylation (Miletic et al., 2015), leading to the trafficking of GluA1 away from pain-modulating neuronal synapses (Tao et al., 2014). Thus, to probe the regulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor GluR1 subunit phosphorylation (GluA1 Ser831 and Ser845) using phosphospecific antibodies, by the stimulation of H_3R (in presence and absence of PF-086087) in primary rat dorsal root ganglion (DRG) neurons using calcium imaging and voltage and current recordings using patch-clamp in the conventional whole-cell and perforated patch configuration approaches. We will also explore the changes in MAPKs, PKCϒ and AMPA receptor phosphorylation (GluA1 Ser831 and Ser845) *in vivo* subsequent to nerve injury and modulated by PF-086087.

Study 3 : Electrophysiology study

To identify mechanisms underlying the effects of H3Rs antagonist PF-0868087 and histamine scavenger inhibition on pain intensity in inflammatory and trigeminal neuropathic orofacial pain, neurons from dorsal root ganglion (DRG) and trigeminal ganglion (TG) of mice will be isolated and processed through voltage and current

recordings using patch-clamp in the conventional whole-cell and perforated patch configuration. This approach will be used with an aim of identifying the role of potassium channel activity modulators (flupirtine, retigabine, etc.) in inducing analgesic effects *via* histaminergic pathways. In addition, spontaneous miniature excitatory and inhibitory currents, and postsynaptic GABA and glutamate currents, will be recorded using solutions described in Telezhkin et al., 2016.

8.4.2 Future clinical studies

Neuropathic pain is a widespread public health issue that affects people all over the world. The majority of current treatments, including opioids, work through the central nervous system, whereas votucalis takes a different approach and mechanism by not entering the central nervous system, potentially reducing severe side effects and preventing opioid addiction.

Study 1.

Currently, available medical treatment for neuropathic pain is insufficient, with more than two-thirds of patients lacking a suitable solution for their comorbid disease and declining quality of life. Most medications used to treat neuropathic pain are systemic (meaning they affect the central nervous system) and are dose restricted due to the severe and debilitating side effects they cause. Scientific evidence suggests that topical medications can be used to treat localized cutaneous neuropathy. Localized treatment has been associated with fewer systemic side effects, drug-drug interactions, and acceptable efficacy (Sommer and Cruccu, 2017). Local peripheral treatments have also been associated with improved compliance of the patient. Previously, Votucalis was used in clinical studies in patients suffering from allergic rhinitis and conjunctivitis. The drug has excellent safety profile and comfort characteristics, and there has been no side effect observed. From that point, using this drug in a selected group of patients suffering from neuropathic pain may represent an important step in controlling this type of condition.

Study 2

Targeting histamine receptors is a well-established treatment route with drugs pointing to specific types of histamine receptors such as H_1R for inflammation and allergy, H_2R

for ulcer and now H_3R for narcolepsy. By contrast, votucalis has a unique different mechanism of action that can likely inhibit all four histamine receptors (H_1R, H_2R, H_3R, and H_4R) by sequestering histamine within the body of the protein thereby preventing histamine binding to its receptors. It is well established that the COVID-19 caused by SARS-CoV-2 *infection* activates mast cells, leading to the release of a wide range of inflammatory mediators including histamine (Conti *et al.*, 2020; Qu *et al.*, 2021). Clinical data from a variety of sources indicate that selective blocking of H_1R (diphenhydramine) and H2R (famotidine) reduce morbidity and mortality associated with COVID-19 (Ennis and Tiligada, 2021; Reznikov *et al.*, 2021; Qu *et al.*, 2021). Based on that, it may be worth conducting an observational clinical pilot experiment on a small group of patients with COVID-19 and used aerosol *administration* of Votucalis, *as this drug* prevented airway hyperreactivity and abrogated peribronchial inflammation, eosinophil recruitment, mucus hypersecretion, and IL-4 and IL-5 secretion also inhibit bronchial hyperreactivity (Chapin *et al.*, 2002; Ryffel *et al.*, 2005; Weston-Davies *et al.*, 2005). Therefore, this pilot clinical experiment may directly lead to the development of a novel therapeutic strategy with urgent clinical benefit and might help for disease prevention, early intervention, or as adjuvant therapy for severe COVID-19.

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Themed Section: New Uses for 21st Century

REVIEW ARTICLE

Histamine, histamine receptors, and neuropathic pain relief

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Histamine, acting via distinct histamine H_1, H_2, H_3, and H_4 receptors, regulates various physiological and pathological processes, including pain. In the last two decades, there has been a particular increase in evidence to support the involvement of H_3 receptor and H_4 receptor in the modulation of neuropathic pain, which remains challenging in terms of management. However, recent data show contrasting effects on neuropathic pain due to multiple factors that determine the pharmacological responses of histamine receptors and their underlying signal transduction properties (e.g., localization on either the presynaptic or postsynaptic neuronal membranes). This review summarizes the most recent findings on the role of histamine and the effects mediated by the four histamine receptors in response to the various stimuli associated with and promoting neuropathic pain. We particularly focus on mechanisms underlying histamine-mediated analgesia, as we aim to clarify the analgesic potential of histamine receptor ligands in neuropathic pain.

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1 | NEUROPATHIC PAIN AND ITS TREATMENT

Neuropathic pain was first defined by the International Association for the Study of Pain as "pain initiated or caused by a primary lesion or dysfunction in the nervous system" (Merskey & Bogduk, 1994). Fourteen years later, Treede et al. (2008) revised this definition and redefined it as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system." In the revised definition of neuropathic pain, two terms have received particular attention. First, the term "disease" which refers to all types of abnormal conditions including inflammation, autoimmune syndromes, and ion channel disorders (channelopathies) replaced the term "dysfunction." Second, to avoid misdiagnosis of neuropathic pain as another type of pain originating from the nervous system, such as spasticity and rigidity of the muscles and bone (e.g., musculoskeletal pain), the term "nervous system" was replaced by the term "somatosensory system" (Finnerup et al., 2016). This revised definition of neuropathic pain describes the nature of this condition more precisely and is, therefore, now widely accepted and approved by the Neuropathic Pain Special Interest Group of the International Association for the Study of Pain. Neuropathic pain can be divided into two subtypes, peripheral or central, based on the anatomical location of the lesion or the disease, within the peripheral nervous system (PNS) or central nervous system (CNS), respectively.

Abbreviations: CaM, calmodulin; DRG, dorsal root ganglion; GSK3β, glycogen synthase kinase 3β; IP_3, inositol triphosphate; KO, knockout; LC, locus coeruleus; PIP_2, phosphatidylinositol 4,5-bisphosphate; PNS, peripheral nervous system; SP, substance P

It is estimated that the worldwide prevalence rate of neuropathic pain in the general population lies between 7% and 10%; however, this figure differs for different countries (van Hecke, Austin, Khan, Smith, & Torrance, 2014). The highest prevalence rates for neuropathic pain were recorded in Canada (17.9%) and in the United States (9.8–12.4%; VanDenKerkhof et al., 2016; Yawn et al., 2009), while a relatively low prevalence rate was noted in Austria and Netherlands (3.3% and 1%, respectively; Bouhassira, Lanteri-Minet, Attal, Laurent, & Touboul, 2008; Gustorff et al., 2008; Harifi et al., 2013). In the United Kingdom, France, and Brazil, it is reported that 7–10% of chronic pain sufferers have been affected by neuropathic pain (Fayaz, Croft, Langford, Donaldson, & Jones, 2016). As a consequence of extended life expectancy, it is predicted that the worldwide prevalence rate of neuropathic pain is likely to increase further, because this type of chronic pain occurs with many common age-related diseases. Neuropathic pain is triggered by a lesion within the somatosensory system, trauma, or to toxic effects of certain medications (Colloca et al., 2017; Yu et al., 2013). Pathological conditions that are responsible for the development of neuropathic pain through injury include metabolic diseases (e.g., diabetic neuropathy), infection (e.g., postherpetic neuralgia), vascular disease (e.g., stroke), trauma (e.g., orofacial neuropathy), and cancer (Campbell & Meyer, 2006). Neuropathic pain is a complex condition that can either be constant or periodic and presents with a range of different symptoms. These symptoms can increase throughout the day with clinically relevant morning–evening differences and can be affected by gender and underlying aetiology (Gilron, Bailey, & Vandenkerkhof, 2013). Sufferers of neuropathic pain have ongoing, spontaneous pain that has a significant negative impact on quality of life and daily functioning, including physical, emotional, and social well-being (Jensen, Chodroff, & Dworkin, 2007).

The mechanisms underlying neuropathic pain are complex and multidimensional. Numerous pathophysiological and biochemical changes cause morphological and functional adaptations in the nervous system, including an increase in excitatory neurotransmitters and neuropeptides, for example, **histamine**, **bradykinin**, 5-HT, and glutamate, which leads to hyperexcitability (Baron, Binder, & Wasner, 2010). Likewise, injured peripheral nerve fibres give rise to an intense and prolonged input of ectopic activity to the CNS and may induce secondary changes to the excitability of the spinal cord dorsal horn neurons (Colloca et al., 2017; Ossipov & Porreca, 2005). These morphological changes and functional adaptations lead to abnormal sensory signs in patients with neuropathic pain presented as, for example, allodynia (pain due to a stimulus that does not normally activate the nociceptive system), hyperalgesia (an increased response to a stimulus that is normally painful), or sensory loss (hypoesthesia). Clinically, neuropathic pain manifests as evoked pain and presents in many ways such as burning, tingling, prickling, shooting, electric shock-like, jabbing, squeezing, spasm, or cold (Rice, Finnerup, Kemp, Currie, & Baron, 2018).

Animal models of neuropathic pain are essential in understanding the plethora of mechanisms that may drive neuropathic pain, allowing the field to identify potential therapeutic targets for the effective management of this condition. Extensive research in the pain field has developed and characterized a wide variety of animal models of neuropathic pain. The four most commonly used models, also adopted in some studies discussed in this review, are chronic constriction injury (Bennett & Xie, 1988), partial sciatic ligation (Seltzer, Dubner, & Shir, 1990), spinal nerve ligation (Kim & Chung, 1992), and spared nerve injury (Decosterd & Woolf, 2000) models. These models aim to simulate some of the clinical features of neuropathic pain, in the preclinical setting (e.g., allodynia and hyperalgesia), because a large proportion of peripheral neuropathic pain models, which are currently used in research, share alterations in hindlimb cutaneous sensory thresholds following partial injury of a peripheral (usually sciatic) nerve. This is associated with the development of neuropathic pain symptoms, such as hyperalgesia and allodynia (Ma et al., 2003; Wall et al., 1979). There are also limitations associated with the use of animal models of neuropathic pain. These limitations are linked to challenges associated with (a) reliable and objective measures of behavioural responses to noxious stimuli, since animals cannot self-report and the experimenter can be biased, (b) appropriateness of the outcome measures, for example, sleep disturbance to reflect spontaneous pain, and (c) complexity of mechanisms underlying the development of neuropathic pain and their relevance to humans (Colleoni & Sacerdote, 2010).

Despite advances in the understanding of the underlying causes and mechanisms leading to the development and maintenance of neuropathic pain, 40% of Europeans who suffer from chronic pain did not achieve satisfactory pain control (Breivik, Collett, Ventafridda, Cohen, & Gallacher, 2006), and to date, no medication has shown long-term efficacy and tolerability for neuropathic pain conditions. A significant contributing factor to these limited therapeutic strategies is that neuropathic pain has different aetiology and pathophysiology to any other type of chronic pain, making the management of this type of chronic pain particularly difficult and challenging (Finnerup et al., 2015). Consequently, there is a pressing need for the identification of new therapeutic strategies to improve management of neuropathic pain that will directly improve the outcome for pain sufferers.

The histamine system has been a target for multiple therapeutic interventions. Recently, growing evidence has supported the use of selective ligands of histamine **H_3 receptor** and **H_4 receptor** for the treatment of neuropathic pain (Bhowmik, Khanam, & Vohora, 2012; Chaumette et al., 2018; Popiolek-Barczyk et al., 2018; Sanna, Mello, Masini, & Galeotti, 2018). Approval in the European Union for the use of **pitolisant** (Wakix™), an antagonist/inverse agonist of H_3 receptors for the treatment of narcolepsy (Kollb-Sielecka et al., 2017), presents an opportunity to explore its clinical use for other conditions. Consequently, it seems an appropriate time to reconsider the histamine system as a therapeutic target for the management of neuropathic pain. This review aims to summarize the most recent findings on the role of histamine and its effects, mediated by different subtypes of histamine receptors, on neuropathic pain, with particular regard to the mechanisms underlying histamine-mediated analgesia.

2 | HISTAMINE, HISTAMINE RECEPTORS, AND PAIN

2.1 | Histamine and pain

Histamine (2-(4-imidazolyl)-ethylamine), one of the most extensively studied amino acid-derived neurotransmitters in the CNS and PNS, is involved in various physiological and pathological processes, including sleep-waking cycle, homeostasis, synaptic plasticity, and learning

(Panula et al., 2015; Parsons & Ganellin, 2006; Pini, Obara, Battell, Chazot, & Rosa, 2016). Histamine is synthesized from the amino acid L-histidine through oxidative decarboxylation via catalysis with the histidine decarboxylase enzyme (Bodmer, Imark, & Kneubuhl, 1999; Haas, Sergeeva, & Selbach, 2008) and is arguably the most pleiotropic molecule in the human and animal body, being present in many cell types (Lindskog, 2017). Histamine is released by neuronal and non-neuronal sources and is responsible for many physiological processes, including the contraction of smooth muscles of the lungs, uterus, and intestine, secretion of gastric acid in the stomach, and vasodilation, and modulation of heart rate and contractility (Parsons & Ganellin, 2006). Histamine also functions as a neurotransmitter, within the nervous system, regulating a variety of body functions, such as temperature control, memory, wakefulness, and pain sensation (Panula & Nuutinen, 2013).

Histamine is a key mediator in the processing of nociceptive information, acting in an antinociceptive manner in the CNS while, conversely, in a nociceptive manner in the PNS (Khalilzadeh, Azarpey, Hazrati, & Vafaei Saiah, 2018). In the PNS, histamine is released in response to tissue injury/damage, and, through the sensitization of polymodal nociceptors resulting in increased firing rates, it contributes to the generation of pain hypersensitivity. In neuropathic pain, histamine released in the periphery by mast cells has been shown to play an important role in the development of hypersensitivity following nerve injury. This pathological process is associated with recruitment of macrophages and neutrophils, and as histamine is a powerful chemoattractant of mast cells, it regulates this recruitment (Smith, Haskelberg, Tracey, & Moalem-Taylor, 2007; Zuo, Perkins, Tracey, & Geczy, 2003). Interestingly, it was also observed that peripherally acting histamine could interact with mechanisms underlying pruritus (itch) and pain. Findings suggest that low concentrations of histamine, acting on sensory neurons, produce pruritus with a high concentration leading to pain (Baron, Schwarz, Kleinert, Schattschneider, & Wasner, 2001; Hough & Rice, 2011; LaMotte, Simone, Baumann, Shain, & Alreja, 1987; Parsons & Ganellin, 2006; Pini et al., 2016; Simone, Alreja, & LaMotte, 1991). There is also evidence to show that histamine-induced itch can convert into pain associated with neuropathic hyperalgesia (Baron et al., 2001). Indeed, multiple itch pathways were identified indicating the presence of distinct itch-generating types of neuron, one responsible for transmitting itch sensation and the other, ultimately, for transmitting pain (Usoskin et al., 2015). In contrast to histamine activity in PNS, multiple behavioural studies have shown that histamine injected directly into the various brain areas (e.g., somatosensory cortex or hippocampus) attenuated pain (Erfanparast, Tamaddonfard, Farshid, & Khalilzadeh, 2010; Tamaddonfard & Hamzeh-Gooshchi, 2014).

Histamine exerts its effects via four distinct GPCR subtypes: H_1, H_2, H_3, and H_4 receptors. These receptors differ in their pharmacology and signal transduction properties (Panula et al., 2015; Parsons & Ganellin, 2006; Simons & Simons, 2011). Thus, histamine has differential effects on neuropathic pain dependent upon the histamine receptor subtype it is bound to. As mentioned previously, this review aims to summarize histamine-mediated effects on neuropathic pain. Therefore, the next sections of this review focus on mechanisms underlying histamine-mediated analgesia.

2.2 | Histamine receptors and pain

Excitatory histamine receptor signalling in nociceptive pathways is associated with increased pain symptoms (Gangadharan & Kuner, 2013; Mobarakeh et al., 2000), whereas inhibition of histamine receptor signalling predominantly causes neuroprotective and antinociceptive effects (Bhowmik et al., 2012; Chazot & Care, 2005; Popiolek-Barczyk et al., 2018). Different subtypes of histamine receptors are expressed in both presynaptic and postsynaptic neuronal membranes (Brown, Stevens, & Haas, 2001; Parsons & Ganellin, 2006; Zhang et al., 2013). Presynaptic histamine receptors function as autoreceptors or heteroreceptors providing either positive or negative feedback regulation of neurotransmitter release from the axon terminals into the synaptic cleft (Nieto-Alamilla, Marquez-Gomez, Garcia-Galvez, Morales-Figueroa, & Arias-Montano, 2016). It could be concluded that the resultant excitatory or inhibitory physiological effect of histamine receptors depends on the action of the neurotransmitter and the subsequent downstream cascade.

Specificity of localization of histamine receptors in different parts of the nervous system, on either presynaptic or postsynaptic membranes, is determined by their physiological relevance (Parsons & Ganellin, 2006). Among the four subtypes of histamine receptors, H_1 and H_2 receptors are predominantly identified postsynaptically (Brown et al., 2001; Connelly et al., 2009; Zhang et al., 2013), with the location of the H_4 receptor requiring further investigation (Connelly et al., 2009). Cross-desensitization and agonist-induced heterodimerization of H_1 and H_2 receptors (Alonso et al., 2013) may suggest a possible partnership between histamine receptors. Expression of H_3 receptors was initially reported as exclusively presynaptic in the rat cerebral cortex (Arrang, Garbarg, & Schwartz, 1983; Clark & Hill, 1996), while postsynaptic expression of H_3 receptors could not be completely excluded (Nieto-Alamilla et al., 2016). H_3 receptors are predominantly expressed in neurons and, together with H_4 receptors, have higher affinity (nM range) for histamine than H_1 and H_2 receptors (μM range; Parsons & Ganellin, 2006). Expression of H_3 and H_4 receptors on the opposite sides of the synaptic cleft may contribute to their effects in neuropathic pain, although the neuronal topology of the H_4 receptor still remains controversial. The use of selective ligands for histamine receptors has led to a better understanding of the physiological and pathophysiological roles of these receptors. The next section summarizes the effects produced by histamine receptor ligands on neuropathic pain.

Besides their presynaptic or postsynaptic localization, the physiological effects of histamine receptors are, to a great extent, determined by the type of guanine nucleotide-binding proteins (G-proteins) to which they are coupled (Leung & Wong, 2017). The difference in underlying signalling pathways may directly determine the effect on pain perception produced by selective ligands, even when they act at the same histamine receptor, as described below.

2.2.1 | H_1 receptor

H_1 receptors are excitatory receptors, which couple with G_q-type proteins, leading to downstream activation of PLC and hydrolysis of

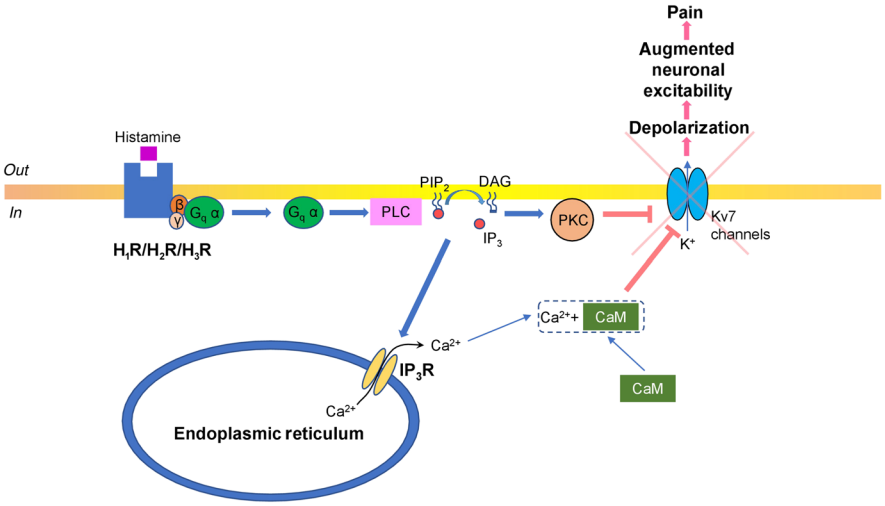

FIGURE 1 Diagram illustrating histamine receptor signalling—G_q pathway. Histamine binds to histamine receptors (H_1, H_2, or H_3 receptor subtypes) that are coupled with the G_q-type protein. G_q α subunit activates PLC which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2), subsequently producing DAG, that remains in the inner leaflet of the plasma membrane activating PKC, and water soluble inositol triphosphate (IP_3), which binds to **inositol triphosphate sensitive receptor** (IP_3R) and stimulates Ca^{2+} release from endoplasmic reticulum. This intracellular Ca^{2+} forms a complex with calmodulin (CaM) Ca^{2+}CaM and induces PKC-dependent phosphorylation. This suppresses the activity of potassium voltage-gated channels type 7 (K_v7 channels), which depolarizes the neurons, and leads to the augmentation of neuronal excitability, which manifests as increased pain symptoms

phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce DAG and **inositol triphosphate** (IP_3). DAG subsequently activates PKC at the membrane, while IP_3 diffuses and binds to IP_3 receptors on the endoplasmic reticulum to mobilize stored calcium (Ca^{2+}). These changes cause PIP_2 depletion and increased intracellular concentration of Ca^{2+}. This increased concentration of Ca^{2+} activates PKC-dependent phosphorylation and forms a complex with **calmodulin** (Ca^{2+}CaM), both of which suppress **potassium voltage-gated type 7 (Kv7) channels** (Figure 1), leading to depolarization and increased nociception (Brown & Passmore, 2009; Chen, Li, Hiett, & Obukhov, 2016).

2.2.2 | H_2 receptor

H_2 receptors are postsynaptic, predominantly associated with **AC** via coupling to G_s and, in a similar fashion to H_1 and H_3 receptors, G_q proteins, both pathways initiating excitatory downstream signalling. Thus, H_2 receptor inhibition yielded efficient antinociceptive effects (Mojtahedin, 2016). G_s α subunit stimulates AC with consequent augmented production of cAMP and consequent activation of **PKA** (Brown et al., 2001). Elevated cAMP concentration up-regulates PKA, which as reported for hippocampal neurons, could activate ligand gated **α-AMPA receptors** with resultant depolarization and increase of neuronal firing (Park et al., 2016). Also, as it was reported for after-hyperpolarization in enteric neurons, PKA inhibits small conductance **Ca^{2+}-activated potassium (KCa) channels** with resultant augmented neuronal excitability (Figure 2; Vogalis, Harvey, & Furness, 2003).

2.2.3 | H_3 receptor

Presynaptic H_3 receptors are coupled with G_i (AC inhibitory) proteins (Nieto-Alamilla et al., 2016; Schlicker & Kathmann, 2017). G_i α subunit-mediated AC inhibition results in a decreased intracellular concentration of cAMP and subsequent down-regulation of PKA (Nieto-Alamilla et al., 2016). In sympathetic and sensory neurons, it

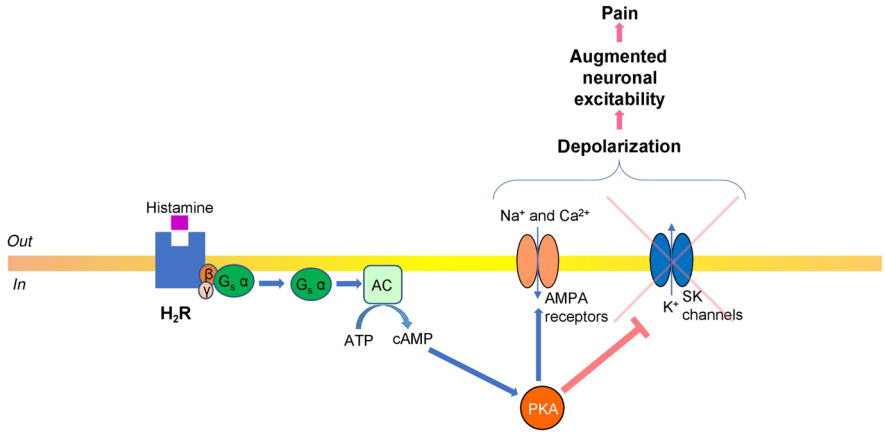

FIGURE 2 Diagram illustrating histamine receptor signalling—G_s pathway. Histamine binds to the H_2 receptor subtype that is coupled with the G_s-type protein. G_s α subunit activates AC, with subsequent production of cAMP, which then stimulates PKA. PKA-dependent phosphorylation activates ligand gated α-AMPA receptors, which open and facilitate influx of Na^+ and, less commonly, Ca^{2+}. PKA also suppresses K^+ efflux through small conductance Ca^{2+}-activated potassium channels (SK channels). Both the activation of AMPA receptors and inhibition of SK channels depolarize the neurons, with consequent augmentation of their excitability and increased pain signalling

was reported that H_3 receptor activation stimulated dissociation of G_i β and γ subunits from G_i α subunit, which then inhibited voltage-gated Ca^{2+} influx through N-, P-, and Q-type Ca^{2+} channels (Zamponi & Currie, 2013), and stimulated **G protein-coupled inwardly rectifying potassium (Kir) channels** (Luscher & Slesinger, 2010). Both mechanisms could hyperpolarize presynaptic neurons, reduce neuronal excitability, and produce pain relief (Figure 3).

Besides the G_i pathway, postsynaptic activation of H_3 receptors was reported to stimulate PLC in a subpopulation of striatal neurons, with subsequent activation of the IP_3 pathway followed by increased intracellular concentrations of Ca^{2+} (Rivera-Ramírez et al., 2016). Thus, it is analogous to the mechanisms described for H_1 /H_2 receptors coupled to G_q proteins (Figure 1). Similarly, the H_1 and H_2 receptor G_q cascade PLC signalling pathways modulate neuronal excitability with resultant potential facilitation of pain sensitivity. Furthermore, H_3 receptor activation was established to inhibit **glycogen synthase kinase 3β** (GSK3β) and MAPK/**ERK** pathways (Bongers et al., 2007; Morita, Aida, & Miyamoto, 1983). These effects are translated via G_i β and γ subunits (Lai et al., 2016), which up-regulate the **PI3K** pathway with subsequent production of **phosphatidylinositol 3,4,5-triphosphate** (PIP_3) from PIP_2, which results in the recruitment of **PKB** (Akt). PKB is initially activated by **phosphoinositide-dependent kinase 1** (PDK1) and **mammalian target of rapamycin** complex 2-dependent phosphorylation (Dzamko, Zhou, Huang, & Halliday, 2014). PKB, via phosphorylation, inactivates GSK3β, which decreases neuronal excitability (Paul et al., 2016), inhibits neuronal inflammation (Maixner & Weng, 2013), and, subsequently, relieves pain. PKB-dependent phosphorylation that activates the MAPK/ERK cascade was also reported to be a highly efficient neuroprotective mechanism for chronic inflammatory and neuropathic pain (Cruz & Cruz, 2007; Figure 3).

2.2.4 | H_4 receptor

The role of H_4 receptors in the nervous system is poorly understood (Schneider & Seifert, 2016). H_4 receptors are known to be coupled to G_i proteins, and their downstream pathways are postulated to be similar to those described for H_3 receptors (Figure 3). Compared to the other three types of histamine receptors, the H_4 receptor is not expressed abundantly in the CNS and PNS. By quantitative single-cell Ca^{2+} imaging, it was demonstrated that histamine induces a Ca^{2+} increase in a subset of sensory neurons (3–10%) via activation of the H_1 and H_4 receptors as well as inhibition of the H_3 receptor. It is assumed that the decreased threshold in response to H_3 receptor antagonism, which accounts for the analgesic effect of H_3 receptor antagonists, activates H_1 and H_4 receptors on sensory neurons, which

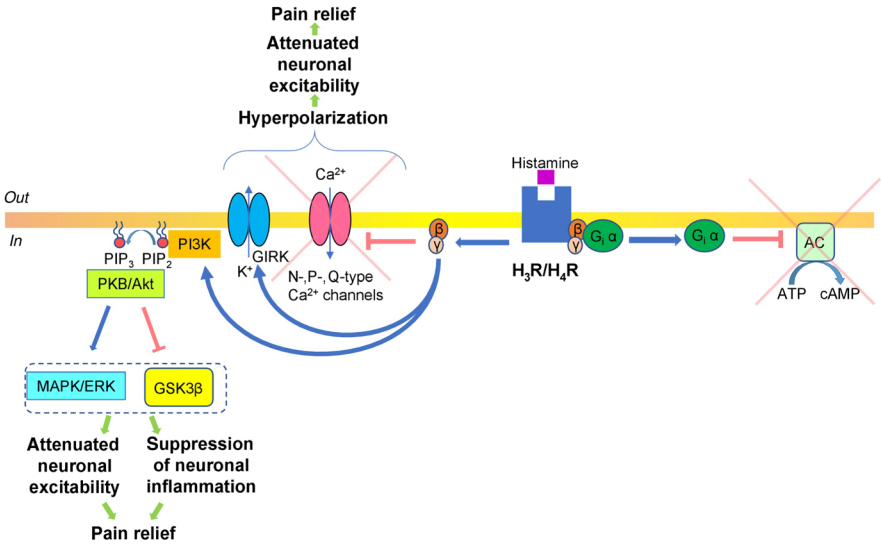

FIGURE 3 Diagram illustrating histamine receptor signalling—G_i pathway. Histamine binds to the histamine receptors (presynaptic and postsynaptic H_3 or H_4 receptor subtypes) that are coupled with G_i-type protein. The G_i α subunit inhibits AC with subsequent suppression of cAMP production and inhibition of PKA activity. Also, G_i β and γ subunits can inhibit Ca^{2+} influx through voltage-gated N-, P-, and Q-type Ca^{2+} channels and stimulate G protein-coupled inwardly rectifying potassium (GIRK) channels, with resultant K^+ efflux. Both effects on N-type Ca^{2+} and GIRK channels result in the development of hyperpolarization, attenuation of neuronal excitability, and resultant pain relief. Besides G_i α subunit effects, H_3 receptor activation could produce analgesic effects through G_i β and γ subunits, which up-regulate the PI3K pathway, with the subsequent production of phosphatidylinositol 2,4,5-trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_3 recruits PKB (Akt), which phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β). In parallel, PKB (Akt)-dependent phosphorylation additionally activates the MAPK/ERK cascade. The action on both GSK3β and MAPK/ERK decreases neuronal excitability, inhibits mechanisms of neuronal inflammation, and, therefore, produces pain relief

in turn results in the excitation of histamine-sensitive afferents and, therefore, may result in a modulation of pain sensitivity (Rossbach et al., 2011).

3 | HISTAMINE RECEPTOR LIGANDS AND NEUROPATHIC PAIN

3.1 | H_1 and H_2 receptor ligands and neuropathic pain

The best-known roles for the H_1 receptor are regulation of vasodilation and bronchoconstriction on multiple cell types, including endothelial and smooth muscle cells, while the H_2 receptor is primarily involved in the modulation of gastric secretion on parietal cells (Barocelli & Ballabeni, 2003; Simons, 2003). There is also evidence for their expression in the nervous system, where they regulate some neuronal functions (Haas et al., 2008; Kashiba, Fukui, & Senba, 2001; Murakami et al., 1999).

Both H_1 and H_2 receptors have been implicated in the role of histamine in nociception and chronic pain (Table 1). Interestingly, with the discovery of H_1 and H_2 receptor ligands in the 1950s, controlled clinical studies using these H_1 and H_2 receptor antagonists reported mild analgesic activity and their potential as analgesic adjuvants, particularly in conditions where pain was induced by histamine. Most of the clinical studies focused on **diphenhydramine** (first generation H_1 receptor antagonist) and showed its analgesic potential in the treatment of dysmenorrhea, atypical head and face pain, trigeminal neuralgia, and thalamic syndrome (Rumore & Schlichting, 1986). In addition, diphenhydramine, when combined with opioids, showed its potential as an analgesic adjuvant in refractory cancer pain (Santiago-Palma, Fischberg, Kornick, Khjainova, & Gonzales, 2001). In addition to clinical evidence for the analgesic potential of H_1 and H_2 receptor antagonists, preclinical studies identified the expression of H_1 and H_2 receptors in nociceptive pathways and, therefore, further supported the roles of H_1 and H_2 receptors in the regulation of pain. There are limited anatomical data available for H_2 receptors, despite the report of H_2 receptor mRNA expression in human spinal cord (Murakami et al., 1999). The potential involvement of H_1 receptors in the modulation of neuropathic pain has been investigated more extensively. In studies using in situ hybridization techniques in the guinea pig, the H_1 receptor mRNA was shown to be expressed in about 15–20% of the central trigeminal and lumbar dorsal root ganglion (DRG) neurons. These sensory neurons are fundamental to nociceptive processes, potentially responding to histamine by acting on H_1 receptors. These neurons are exclusively small in size and coexpress isolectin B4, but not **substance P** (SP) or **CGRP**, suggesting characteristics of unmyelinated C-fibres involved in acute nociception. Interestingly, the potential role of H_1 receptors in the regulation of neuropathic pain sensitivity can be explained by the marked (up to fourfold) increase in H_1 receptor expression in the mainly small-sized DRG neurons, 1–5 days after a crush injury of the sciatic nerve. Moreover, this study showed new characteristics of peptidergic (SP/CGRP) sensory neurons not detected prior to nerve injury, suggesting that the sensory modalities evoked by histamine acting via H_1 receptors in normal and neuropathic pain states may result in different effects. This demonstration of the potential up-regulation of H_1 receptor number in injured afferent nerves further supports the involvement of H_1 receptors in the regulation of neuropathic pain hypersensitivity, presumably expressed on unmyelinated C-fibres (Kashiba et al., 2001). The earliest electrophysiological study to probe the histaminergic system in neuropathic pain transmission reported that daily injections of **astemizole**, a CNS-sparing H_1 receptor antagonist, acting via peripheral histamine-sensitive C-fibres, blocked ectopic spontaneous discharges from the neuroma and suppressed autotomy following sciatic and saphenous neurectomy (Seltzer et al., 1991; Yu et al., 2013). More recently, i.c.v. injection of histamine blocked mechanical and thermal hypersensitivity associated with neuropathic pain (Sanna et al., 2015; Wei et al., 2016). However, these pain-modulatory effects of histamine can vary depending on the dose of histamine administered i.c.v., due to an action on H_2 receptors and the involvement of adrenoceptors (Wei et al., 2014; Wei et al., 2016).

To further support the role of H_1 and H_2 receptors in the regulation of pain, separate studies using knockout (KO) mice lacking H_1 and H_2 receptors demonstrated that these mice displayed significantly lower responses to nociceptive stimuli when compared to their wild-type controls (Mobarakeh et al., 2002; Mobarakeh, Takahashi, Sakurada, Kuramasu, & Yanai, 2006). Interestingly, the antinociceptive phenotype of H_2 receptor KO mice was relatively less prominent when compared to H_1 receptor KO mice, suggesting a potentially distinct role for these receptors in the modulation of pain. Indeed, behavioural studies using a model of neuropathic pain, induced by the partial ligation of the sciatic nerve, showed that the CNS-permeable H_1 receptor antagonist **mepyramine**, but not the H_2 receptor CNS-sparing antagonist **cimetidine**, blocked the effects of histidine on neuropathic pain hypersensitivity and spinal microglia activity (Yu et al., 2016). In addition, Jaggi et al. (2017) suggested that the H_1 receptor plays a more important role in a vincristine-induced model of neuropathic pain, when compared to H_2 receptors. However, Khalilzadeh et al. (2018) observed different behavioural effects upon tibial nerve transection-induced neuropathic pain with respect to the extent of brain penetration of the ligands, in a study focused on centrally active and centrally sparing H_1 and H_2 receptor antagonists. Specifically, both **chlorpheniramine**, a centrally and peripherally active H_1 receptor antagonist, and **fexofenadine**, an H_1 receptor, centrally sparing antagonist, were found to profoundly decrease the mechanical hypersensitivity associated with the development of neuropathic pain. In contrast, while **ranitidine**, a widely used centrally permeable H_2 receptor antagonist, also improved mechanical hypersensitivity, **famotidine**, a centrally sparing H_2 receptor antagonist, was ineffective. These results indicate that both blood brain barrier penetrating and poorly penetrating histamine H_1 receptor antagonists can block neuropathic pain hypersensitivity, but only the blood brain barrier penetrating histamine H_2 receptor antagonist can generate an analgesic effect in neuropathic pain. In line with this observation, histamine-induced mechanical hypersensitivity

TABLE 1 A summary of the effects produced by histamine receptor ligands in animal models of neuropathic pain

Drug	Model	Strain	Test	Effect	Reference
H_1 antagonists					
Astemizole	Neurectomy	Sabra rats	Score	i.p. suppressed autotomy	Seltzer, Paran, Eisen, & Ginzburg, 1991
Chlorpheniramine	TNT	Wistar rats	Acetone von Frey Double plate Open field	i.p. reduced allodynia and prevented cold plate avoidance behaviour	Khalilzadeh et al., 2018
	PLSN	Wistar rats	Hargreaves Randall-Selito	i.p. suppressed and alleviated hyperalgesia	Zuo et al., 2003
Fexofenadine	TNT	Wistar rats	Acetone von Frey Double plate Open field	p.o. reduced allodynia and prevented cold plate avoidance behaviour	Khalilzadeh et al., 2018
Loratadine	Peripheral axotomy	Sprague–Dawley rats	Score of autotomy	i.p. did not block analgesic effects of histidine on pain behaviour but alone suppressed autotomy	Yu et al., 2013
Mepyramine (pyrilamine)	PLSN	Sprague–Dawley rats	von Frey IR laser	Intrathecally, i.c.v. blocked analgesic effects of histidine on pain behaviour	Yu et al., 2016
	SNL	Hannover-Wistar rats	von Frey Radiant heat	Intrathecally did not attenuate the antihypersensitivity effect of histamine	Wei, Viisanen, You, & Pertovaara, 2016
	SNL	Hannover-Wistar rats	von Frey test Radiant heat	Into LC did not attenuate the antihypersensitivity effect of histamine and alone failed to influence pain	Wei, Jin, Viisanen, You, & Pertovaara, 2014
	PLSN	Rats	von Frey	i.c.v was ineffective	Huang, Adachi, Nagaro, Liu, & Arai, 2007
Promethazine	Vincristine induced	Wistar albino rats	Pinprick Acetone Hot plate	i.p. reduced hyperalgesia and allodynia	Jaggi, Kaur, Bali, & Singh, 2017
H_2R antagonists					
Cimetidine	PLSN	Sprague–Dawley rats	von Frey IR laser	Intrathecally, i.c.v. did not block histidine's analgesic effects on pain behaviour	Yu et al., 2016
	PLSN	Wistar rats	Hargreaves Randall-Selito	i.p. suppressed and alleviated hyperalgesia	Zuo et al., 2003
Famotidine	TNT	Wistar rats	Acetone von Frey Double plate Open field	p.o. was ineffective in all tests	Khalilzadeh et al., 2018
	PLSN	Sprague–Dawley rats	von Frey Diode laser	i.p. reduced allodynia and hyperalgesia	Yue et al., 2014

(Continues)

TABLE 1 (Continued)

Drug	Model	Strain	Test	Effect	Reference
Ranitidine	TNT	Wistar rats	Acetone von Frey Double plate Open field	i.p. reduced allodynia and prevented cold plate avoidance behaviour	Khalilzadeh et al., 2018
	Vincristine induced	Wistar rats	Pinprick Acetone Hot plate	i.p. reduced hyperalgesia and allodynia	Jaggi et al., 2017
	PLSN	Rats	von Frey	i.c.v. increased hypersensitivity	Huang et al., 2007
Zolantidine	SNL	Hannover-Wistar rats	von Frey Radiant heat	i.t. attenuated the antihypersensitivity effect of histamine	Wei et al., 2016
	SNL	Hannover-Wistar rats	von Frey Radiant heat	Into LC attenuated histamine's analgesic effect but alone failed to influence pain	Wei et al., 2014
H_3R antagonists and H_3R antagonists/inverse agonist					
A-960656	SNL	Hannover-Wistar rats	von Frey Radiant heat	Into LC reduced hypersensitivity	Wei et al., 2014
	SNL	Hannover-Wistar rats	von Frey Radiant heat	i.t. reduced hypersensitivity	Wei et al., 2016
	SNL	Sprague–Dawley rats	von Frey	p.o. reduced hypersensitivity	Cowart et al., 2012
Ciproxifan	SNI	BL6 mice	Hargreaves Dynamic plantar aesthesiometer	i.p. was ineffective	Zhang et al., 2012
E-162	CCI	Swiss CD1 mice	von Frey Cold plate Tail-flick	i.p. reduced hypersensitivity	Popiolek-Barczyk et al., 2018
GSK189254	CCI	Random-hooded rats	von Frey Randall-Selito	p.o. reduced allodynia and hyperalgesia	Medhurst et al., 2008
	SNL	Sprague–Dawley rats	von Frey	i.p. reduced allodynia	Hsieh et al., 2010
GSK334429	CCI	Random-hooded rats	von Frey Randall-Selito	p.o. reduced allodynia and hyperalgesia	Medhurst et al., 2008
Pitolisant	SNI	BL6 mice	Hargreaves Dynamic plantar aesthesiometer	i.p. was ineffective, high doses increased thermal but not mechanical hypersensitivity	Zhang et al., 2012
S38093	CCI and oxaliplatin induced	Sprague–Dawley rats	Randall-Selito Tail-immersion (10°C)	p.o. reduced hypersensitivity	Chaumette et al., 2018

(Continues)

TABLE 1 (Continued)

Drug	Model	Strain	Test	Effect	Reference
ST-889	SNI	BL6 mice	Hargreaves Dynamic plantar aesthesiometer	i.p. was ineffective	Zhang et al., 2012
Thioperamide	PLSN PLSN	Rats Sprague–Dawley rats	von Frey Randall-Selito	i.c.v. increased but i.p. reduced hypersensitivity i.p. increased hypersensitivity	Huang et al., 2007 Smith et al., 2007
H_4R agonists					
ST-1006	SNI	CD1 mice	Hargreaves Dynamic plantar aesthesiometer	i.c.v. reduced allodynia and hyperalgesia	Sanna et al., 2015
VUF-8430	SNI	CD1 mice	Hargreaves Dynamic plantar aesthesiometer	i.c.v. reduced allodynia and hyperalgesia	Sanna et al., 2015
	SNI	CD1 mice	Hargreaves Dynamic plantar aesthesiometer	i.t.reduced allodynia and hyperalgesia	Sanna, Lucarini, et al., 2017
	PLSN	Sprague–Dawley rats	Randall-Selito	i.p. reduced hypersensitivity	Smith et al., 2007
H_4R antagonists					
JNJ7777120	PLSN	Sprague–Dawley rats	Randall-Selito	i.p. increased hypersensitivity	Smith et al., 2007
	SNL	Sprague–Dawley rats	von Frey	i.p. reduced allodynia	Hsieh et al., 2010
JNJ10191584	SNI	CD1 mice	Hargreaves Dynamic plantar aesthesiometer	p.o. blocked the analgesic effect of i.c.v. ST-1006 and VUF-8430 and was ineffective alone	Sanna et al., 2015
	SNI	CD1 mice	Hargreaves Dynamic plantar aesthesiometer	p.o. blocked the analgesic effect of i.t. VUF-8430 and was ineffective alone	Sanna et al., 2015
TR-7	CCI	Swiss CD1 mice	von Frey Cold plate Tail-flick	i.p. reduced hypersensitivity	Popiolek-Barczyk et al., 2018

Abbreviations: CCI, chronic constriction nerve injury; LC, locus coeruleus; PLSN, partial ligation of sciatic nerve; SNI, spared nerve injury; SNL, spinal nerve ligation; TNT, tibial nerve transection.

was prevented by spinal pretreatment with zolantidine, a brain penetrating H_2 receptor antagonist, as well as localized peripheral administration of cimetidine (H_2 receptor antagonist) and chlorpheniramine (H_1 receptor antagonist) into the plantar side of the hindpaw (Zuo et al., 2003).

Taken together, these results indicate that the brain histamine, acting particularly via central H_1 and H_2 receptors, may be involved in the modulation of neuropathic pain. These studies consistently support the idea that CNS-permeable H_1 and H_2 receptor antagonists may potentially be used as analgesics for patients with neuropathic pain. The involvement of central H_2 receptors in the regulation of neuropathic pain hypersensitivity was also demonstrated in studies where histamine (presumably postsynaptically- induced) facilitated mechanical hypersensitivity mediated by **NMDA** receptors as well as, in a dose-dependent manner, **Nav1.8** channel expression in primary afferent neurons in the sciatic nerve and L4/L5 DRG (Wei et al., 2016; Yue et al., 2014). While sodium channels are responsible for the development and maintenance of neuropathic pain (Ossipov & Porreca, 2005; Yue et al., 2014), the above studies highlight the importance of histamine acting via H_2 receptors in the regulation of mechanisms associated with neuropathic pain states. The influence of the H_2 receptor on non-neuronal cells (mast cells) is discussed later in this review.

3.2 | H_3 receptor ligands and neuropathic pain

H_3 receptors are mostly presynaptic, expressed as autoreceptors on histaminergic neurons involved in the negative feedback control of histamine levels (Arrang et al., 1983; Hough & Rice, 2011), while H_3 heteroreceptors on postsynaptic nonhistaminergic neurons also regulate negatively the release of neurotransmitters, such as **ACh**, **dopamine**, 5-HT, and **noradrenaline** (Blandina, Munari, Giannoni, Mariottini, & Passani, 2010; Gemkow et al., 2009; Giannoni et al., 2010). Since the cloning of H_3 receptors (Lovenberg et al., 1999), there has been an increased interest within the pharmaceutical industry in developing ligands for this receptor to target several diseases, including neuropathic pain. This interest was strongly fuelled by the report of H_3 receptor expression in nociceptive pathways, suggesting its functional involvement in the regulation of nociceptive transmission (Cannon et al., 2007). Indeed, the histamine H_3 receptor consists of several functional isoforms expressed in both the CNS and PNS, particularly along the ascending nociceptive pathway and descending pain-control pathway, that are critical for the processing of nociceptive information. Within the CNS, this receptor has been found in various brain areas, such as thalamus, hypothalamus, prefrontal cortex, and periaqueductal grey area (Drutel et al., 2001), and in the spinal cord (Cannon et al., 2007; Heron, Rouleau, Cochois, Pillot, & Schwartz, 2001; Medhurst et al., 2008). In the periphery, the expression of H_3 receptors has been identified in DRG, superior cervical ganglia, and dermal tissues (Cannon et al., 2007; Medhurst et al., 2008).

However, while the localization of H_3 receptors strongly suggests its functional involvement in the regulation of nociceptive transmission, pharmacological studies using agonists and antagonists of H_3 receptors are confusing as these drugs have different effects on the nociceptive threshold depending on the pain model used, the nociceptive stimulus selected, together with the affinity and selectivity for the histamine receptors, and the dose and routes of administration (Huang et al., 2007; Smith et al., 2007). Several studies have reported inhibitory effects on pain following activation of H_3 receptors using agonists (Cannon et al., 2003; Hasanein, 2011). The involvement of H_3 receptors in neuropathic pain has been implicated using a range of H_3 receptor antagonists/inverse agonists (Table 1). The antagonism at H_3 receptors results in reduced mechanical and cold hypersensitivity associated with neuropathic pain (chronic constriction injury model or spinal nerve ligation model) as shown in studies using E-162 or GSK189254, selective H_3 receptor antagonists, where its strong analgesic effect was observed after a single systemic (i.p.) dose (Hsieh et al., 2010; Popiolek-Barczyk et al., 2018). In addition, repeated, orally delivered doses of GSK189254, **GSK334429**, S38093, and A-960656, selective H_3 receptor antagonists/inverse agonists, significantly reduced paw withdrawal threshold to mechanical stimuli or elicited an analgesic effect in the vocalization test of neuropathic pain (chronic constriction injury model or spinal nerve ligation model), showing comparable efficacy to **pregabalin** or **gabapentin**, which are both used clinically as first-line treatments (Chaumette et al., 2018; Cowart et al., 2012; Medhurst et al., 2008). The analgesic efficacy of S38093 was also confirmed in other models of neuropathic pain with different aetiologies, such as diabetic and chemotherapeutic agent-induced neuropathy, where the drug again showed analgesic potency similar to pregabalin and gabapentin (Chaumette et al., 2018). Analgesia induced by the blockade of the H_3 receptor is possibly the result of the regulation of histamine levels in the CNS, as depolarization activates histamine synthesis in nerve endings, a process that is controlled by H_3 autoreceptors (Arrang et al., 1983; Hough & Rice, 2011). Indeed, Wei et al. (2016) proposed that blocking the autoinhibitory H_3 receptor on histaminergic terminals in the pontine locus coeruleus (LC), which receives efferent projections from the histaminergic tuberomammillary nucleus, facilitated endogenous release of histamine leading to neuropathic hypersensitivity inhibition through the regulation of descending noradrenergic pathways. In addition, there is accumulating evidence to support the idea that the analgesic effects of H_3 receptor antagonists/inverse agonists in neuropathic pain can be partially mediated by **α2 adrenoceptor** desensitization (induced by H_3 receptor antagonists/inverse agonists) in the LC and spinal cord. This suggests an inhibitory role for the central heteroreceptor noradrenergic transmission in the efficacy of H_3 receptor antagonists/inverse agonists. In agreement with this idea, systemic administration of α_2 adrenoceptor agonists or nerve injury-induced activation of these α_2 adrenoceptors decreases the firing activity of LC noradrenergic cells, resulting in the dampening of noradrenaline release in the terminal area (e.g., prefrontal cortex or spinal cord) and promoting neuropathic hypersensitivity by attenuating descending inhibition (Chaumette et al., 2018; Wei et al., 2010; Wei et al., 2014). In contrast, treatment with H_3 receptor antagonists/inverse agonists restores LC and decreases α_2 adrenoceptor activity, respectively, potentially leading to relief in neuropathic pain hypersensitivity

(Chaumette et al., 2018; Wei et al., 2010). To further support this proposed mechanism, it was shown that bilateral lesion of the LC, transection of the spinal cord, or direct injection of a α_2 agonist (fadolmidine) into the LC reversed the antihyperalgesic effect produced by H_3 receptor antagonists, A-960656 or GSK189254 (Chaumette et al., 2018; McGaraughty, Chu, Cowart, & Brioni, 2012; Wei et al., 2014). In line with this, electrophysiological studies performed in anaesthetized animals indicated that, after systemic administration, GSK189254 dose-dependently decreased both evoked and spontaneous firing of wide dynamic range neurons in neuropathic, but not sham-operated rats (McGaraughty et al., 2012). However, analgesia induced by the blockade of the H_3 receptor can also be mediated via H_3 heteroreceptors that regulate other neurotransmitters' release; the blockade of the H_3 receptor is known to increase the release of ACh, dopamine, 5-HT, noradrenaline, and SP in the CNS (Blandina et al., 2010; Gemkow et al., 2009; Giannoni et al., 2010).

Interestingly, the majority of the behavioural observations published indicate that H_3 receptor antagonists/inverse agonists do not produce any antinociceptive effects in *naïve* rodents, suggesting a possibility that H_3 receptors are not involved or tonically activated in nociception (at least in relation to acute mechanical nociception), but are critical for pathological pain states, particularly for mechanical hypersensitivity (Chaumette et al., 2018; McGaraughty et al., 2012). Also, H_3 receptor KO mice showed unaltered response to mechanical pinch (Cannon et al., 2003), and multiple studies suggest modality (mechanical vs. heat) and intensity (preferential responses to low-intensity tail pinch stimulation; Cannon et al., 2003) with specific antinociceptive effects mediated by H_3 receptors. H_3 receptor antagonists/inverse agonists at a dose that produced a significant reduction of mechanical hypersensitivity in neuropathic pain did not attenuate heat hypersensitivity indicating that the antihyperalgesic effect was due to selective depression of spinal sensory rather than motor neurons (Wei et al., 2014; Wei et al., 2016). To support this, in situ hybridization studies revealed H_3 receptor mRNA transcripts in the sensory neurons of the dorsal horn and DRG (Heron et al., 2001). Moreover, receptor autoradiography studies, using [^{3}H] GSK189254, showed specific H_3 receptor binding sites in the dorsal horn of the spinal cord and DRG, confirming these as sites of action of H_3 receptor antagonists within structures receiving histaminergic innervation and are critical for processing of pain information (Medhurst et al., 2008). The modality- and intensity-specific antinociceptive effects of H_3 receptor activation/inhibition may also suggest involvement of a specific population of sensory fibres that regulate mechanical hypersensitivity. In line with this, immunohistochemical studies identified localization of H_3 receptors (confirmed by H_3 receptor KO mice) on medium-size cell bodies in DRG and on small-calibre periarterial, peptidergic Aδ fibres that ramified in dorsal horn laminae I, II, and V and coexpress immunoreactivity for acid-sensing ion channel 3 and 200-kD neurofilament protein. This strongly supports the involvement of H_3 receptors in the regulation of mechanical sensitivity (Cannon et al., 2007). In addition, Medhurst et al. (2007) showed that GSK207040 and GSK334429, selective H_3 receptor antagonists, blocked the secondary mechanical allodynia in the capsaicin-induced model of pain. Secondary mechanical hypersensitivity is known to be exclusively signalled by A-fibres and amplified by sensitized dorsal horn neurons (Magerl, Fuchs, Meyer, & Treede, 2001; Treede & Magerl, 2000). Thus, presumably reducing the sensitivity of H_3 receptor-positive A-fibres with selective H_3 receptor antagonists resulted in a diminished input to the dorsal horn and the subsequent amplification of the A-fibres response, confirming the potential role for H_3 receptors in the modulation of central sensitization processes. In contrast, thermal (heat) hypersensitivity is generally regarded as a sign of the peripheral sensitization of C-fibres, which do not express H_3 receptors (Cannon et al., 2007; Gold & Gebhart, 2010). The only study that reported a significant increase in thermal (radiant heat in Hargreaves test), but not mechanical, threshold in the spared nerve injury model of neuropathic pain used the selective H_3 receptor inverse agonist pitolisant (Wakix™). The drug produced this unexpected effect at a dose 5× higher than its clinically relevant dose, and pharmacological analysis of this effect suggested at least partial involvement of transient receptor potential cation channel subfamily V member 1 (**TRPV1**), without any contribution of H_3 receptors (Zhang et al., 2012). Interestingly, the H_3 receptor antagonist/inverse agonist E-162, at a dose that produced a significant reduction in mechanical hypersensitivity, also attenuated the response to cold in neuropathic pain (Popiolek-Barczyk et al., 2018). The signalling of cool temperatures that become aversive in neuropathic pain is known to be mediated via the **TRPM8** receptor, a member of the TRP channel family (Knowlton et al., 2013). It was reported that the number of TRPM8-positive Aδ-fibres (but not C-fibres) increases after nerve injury (Ji, Zhou, Kochukov, Westlund, & Carlton, 2007); thus, it is possible that H_3 receptor-positive A-fibres are probably sensitive to cooling and may contribute to cold hypersensitivity in neuropathic pain.

The most significant inconsistencies in behavioural outcomes in neuropathic pain can be found in studies on the role of a first-generation imidazole-based molecule, **thioperamide** (H_3 receptor antagonist, H_3 /H_4 receptor inverse agonist), in the regulation of mechanical hypersensitivity in neuropathic pain. On the one hand, blocking H_3 receptors (and H_4 receptors) by thioperamide resulted in a significant enhancement of mechanical hyperalgesia in a rat model of neuropathic pain induced by partial ligation of the sciatic nerve. Specifically, i.c.v. (Huang et al., 2007) or s.c. injection (Smith et al., 2007) of thioperamide directly into the operated hindpaw resulted in a significantly reduced mechanical withdrawal threshold as compared to controls. On the other hand, systemic (i.p.) injection of thioperamide significantly increased mechanical withdrawal threshold indicating an analgesic effect (Huang et al., 2007). The reason for this discrepancy may lie in the drug's dual affinity for both H_3 and H_4 receptors (e.g., the effect of thioperamide on neurotransmitter release in the anterior hypothalamic area of rats is nonreversible by an H_3 receptor agonist, suggesting the involvement of H_4 receptors; Yamamoto, Mochizuki, Okakura-Mochizuki, Uno, & Yamatodani, 1997) and on the behavioural effects resulting from the route (localized vs. systemic) and dose of thioperamide administration. In addition, the involvement of other histaminergic mechanisms of action in the behavioural effects

produced by thioperamide is suggested by the observation that thioperamide increases the density of intact mast cells in the injured nerve (Smith et al., 2007). While nerve injury causes a decrease in mast cell numbers as a consequence of degranulation (Zuo et al., 2003), thioperamide's action leads to an opposite effect that may represent a prevention of mast cell degranulation and stabilization or redistribution of mast cells in the injured nerve that theoretically would result in the inhibition of hyperalgesia, rather than its enhancement. This effect could be linked to the observation that histamine (acting through H_1 and H_3 receptors) inhibits the release of the pro-inflammatory cytokine **TNF-α** from alveolar macrophages (Sirois, Ménard, Moses, & Bissonnette, 2000), and antagonism of H_3 receptors on macrophages resulted in an increase in TNF-α and, subsequent, enhancement of mechanical hyperalgesia (Smith et al., 2007).

Taken together, the interpretation of the thioperamide data is complicated further since the drug has high affinity, not only for H_3 and H_4 receptors but also for **5-HT3 receptors** (Leurs et al., 1995). Studies with more selective H_3 receptor antagonists/inverse agonists suggest that these ligands may be beneficial for the improvement of mechanical and cold hypersensitivity associated with neuropathic pain, particularly given their ability to modulate histamine levels, as well as several neurotransmitters, including ACh, histamine, noradrenaline, dopamine, and SP. However, due to the wide presynaptic and postsynaptic distribution of H_3 receptors throughout the CNS and PNS, more research is certainly needed to clarify the involvement of peripheral, spinal, and brain H_3 receptors in various neuropathic pain states, thus determining their full potential in neuropathic pain.

3.3 | H_4 receptor ligands and neuropathic pain

The H_4 receptor, which has low homology with other histamine receptors, can be primarily found in bone marrow, intestinal tissue, spleen, thymus, and also in various immune cells, such as T cells, mast cells, neutrophils, and eosinophils, showing modulatory effects on these cells, including activation, migration, and production of cytokines and chemokines, suggesting its principal role in the regulation of immune/inflammatory mechanisms (Takeshita, Sakai, Bacon, & Gantner, 2003; Zhu et al., 2001). Interestingly, recent reports also indicate the presence of H_4 receptors on peripheral sensory nerves, in the DRG, with more intense staining of small- and medium-diameter cells, and in the spinal cord, especially laminae I and II (Sanna, Lucarini, et al., 2017; Strakhova et al., 2009). This neuronal localization supports H_4 receptors involvement in the regulation of neuronal function related to the modulation of nociceptive transmission (Sanna, Ghelardini, Thurmond, Masini, & Galeotti, 2017; Sanna, Lucarini, et al., 2017).

The involvement of H_4 receptors in both acute (Galeotti, Sanna, & Ghelardini, 2013) and persistent inflammatory pain (Hsieh et al., 2010) is relatively well documented, and recently, the role of H_4 receptors in the modulation of neuropathic pain was identified in H_4 receptor-KO mice through the observation that these animals, when subjected to neuropathic pain, induced by spared nerve injury of sciatic nerve, showed enhanced hypersensitivity to mechanical and thermal stimuli compared to wild-type controls (Sanna, Ghelardini, et al., 2017). Interestingly, H_4 receptor deficiency does not support a role for H_4 receptors in the physiological maintenance of pain threshold, as H_4 receptor-KO mice did not show any change in thermal or mechanical nociceptive thresholds, suggesting that the H_4 receptor is specifically involved in the regulation of hypersensitivity associated with pathological chronic pain induced by nerve injury (Sanna, Ghelardini, et al., 2017). This observation in H_4 receptor-KO neuropathic mice is particularly important as H_4 receptor mRNA expression in humans and rodents supports their involvement in the regulation of neuronal function, including regulation of neuropathic pain. The controversy around the generation of consistently specific H_4 receptor antibodies highlights the need for cautious interpretation of some of the immunohistochemical outcomes (Beermann, Seifert, & Neumann, 2012; Gutzmer et al., 2012; Schneider & Seifert, 2016). In line with the observation from H_4 receptor KO mice, blockade of H_4 receptors by the specific H_4 receptor antagonist JNJ7777120, injected s.c. directly into the operated hindpaw, resulted in a significant increase in mechanical hyperalgesia compared to controls (Smith et al., 2007). Subsequently, activation of H_4 receptors by localized administration of potent and selective agonists, **ST-1006** (i.c.v.) and **VUF8430** (i.c.v., intrathecally, and s.c. directly into the operated hindpaw), resulted in a significantly reduced mechanical and thermal withdrawal threshold in mice subjected to neuropathic pain induced by spared nerve injury or partial nerve ligation of the sciatic nerve (Sanna et al., 2015; Sanna, Lucarini, et al., 2017; Smith et al., 2007). The analgesia produced by VUF8430 has been shown to be associated with a reduction in neuroinflammation and oxidative stress mediated by neuronal H_4 receptors in the spinal cord and sciatic nerve (Sanna, Lucarini, et al., 2017), and the involvement of H_4 receptors in the behavioural effects produced by ST-1006 and VUF8430 was confirmed with **JNJ10191584**, H_4 receptor antagonist also known as VUF6002, which fully prevented the analgesic effects produced by these H_4 receptor agonists (Sanna et al., 2015).

Interestingly, similar to the H_3 receptor, pharmacological studies using agonists and antagonists of H_4 receptors demonstrate that these drugs can have different effects on the nociceptive threshold depending on the routes of administration and target cells (Popiolek-Barczyk et al., 2018; Sanna et al., 2015). In contrast to the studies above that used H_3 receptor agonists/antagonists after localized application, the antagonism of H_4 receptors produced by systemic administration resulted in the alleviation of mechanical and cold hypersensitivity associated with neuropathic pain (chronic constriction injury model). Studies using TR-7, a selective H_4 receptor antagonist, elicited a strong analgesic effect after a single systemic (i.p.) dose, which was as effective as **morphine**, a gold standard in pain treatment (Popiolek-Barczyk et al., 2018). In addition, JNJ7777120 reduced mechanical hypersensitivity after a systemic (i.p.) administration in neuropathic pain (chronic constriction injury model and spinal nerve ligation model; Hsieh et al., 2010). Given that H_4 receptors are expressed on the immune cells, in addition to the well-documented involvement of H_4 receptors in the regulation of immune/inflammatory mechanisms (Takeshita et al., 2003; Zhu et al., 2001), it is possible that the antinociceptive action of H_4 receptor antagonists, particularly after systemic administration,

may result from a reduction in ongoing inflammatory processes at the site of nerve injury, since the analgesic effect produced by JNJ7777120 was weaker (secondary) than its anti-inflammatory effect (Hsieh et al., 2010). An underlying mechanism may be associated with stabilization of mast cells that are known to regulate the recruitment of neutrophils and macrophages and, subsequently, to modulate the development of hyperalgesia in neuropathic pain (Smith et al., 2007; Zuo et al., 2003). It should be further noted that similar observations have been described for the closely related H_3 receptor.

The H_4 receptor is known to activate the MAPK signalling pathway in mast cells (Desai & Thurmond, 2011). Interestingly, Sanna et al. (2015, 2018) also identified the effect of H_4 receptor stimulation on the activity of the MAPK signalling pathway in neurons. They demonstrated that modulation of this signalling pathway within the neurons of the DRG, spinal cord, and sciatic nerve underpinned H_4 receptor agonist-induced antiallodynic activity. They also revealed that neuropathic pain hypersensitivity observed in H_4 receptor-KO mice is associated with an overactivation of the spinal ERK–**cAMP response element-binding protein** pathway in DβH immunoreactive neurons, supporting a potential association between the noradrenergic system and H_4 receptor-mediated analgesia. In summary, increasing evidence arising from H_4 receptor KO mice and the use of selective ligands support H_4 receptor as an interesting neuronal target for the treatment of chronic, particularly neuropathic, pain.

4 | HISTAMINE AND NON-NEURONAL CELLS IN NEUROPATHIC PAIN

Following peripheral nerve injury, the immune system seems to play a vital role in the development of persistent inflammation and chronic neuropathic pain (Marchand, Perretti, & McMahon, 2005). Non-neuronal astrocytes, satellite glia cells, microglia, and mast cells play key roles in communication between the immune system and the CNS via the production of neuroinflammatory mediators, including histamine, 5-HT, chemokines, and growth factors (Zhuang, Gerner, Woolf, & Ji, 2005). The neuroimmune interactions between these two systems may reflect distinct roles in the development of chronic neuropathic pain (Zhao et al., 2017). Stimulation of H_1 receptors via a PKC/MAPK/**MEK1** signalling pathway has recently been shown to elicit release of the key pro-inflammatory cytokines **IL-1β** and **IL-6** with, subsequent, regulation of **nerve growth factor** release from astrocytes (reviewed recently in Jurič, Kržan, & Lipnik-Stangelj, 2016). Satellite glial cells, prominent in the PNS, including the DRGs, with active roles in persistent neuropathic pain are also known to secrete the cytokine IL-6 in the chronic constriction injury neuropathic pain model, but the identity profile of the histamine receptor in these cells has yet to be established (Dubový, Klusáková, Sviženská, & Brázda, 2010). Activated microglia also release a myriad of pro-inflammatory cytokines, including notably, IL-6, IL-1β, and TNF-α (Kempuraj et al., 2016; Mika, Zychowska, Popiolek-Barczyk, Rojewska, & Przewlocka, 2013). H_3 and H_4 receptor activation of primary and clonal microglia has been shown to inhibit these cytokines (Ferreira et al., 2012).

Mast cells are professional cellular suppliers of histamine and contribute to the histamine-based effects in neuropathic pain. For example, mast cell depletion prevented mechanical allodynia in a mouse model of postoperative pain (Kaur, Singh, & Jaggi, 2017). Recently, it was shown that the administration of **azelastine hydrochloride**, a second-generation H_1 receptor antagonist and mast cell stabilizer, blocked the development of mechanical allodynia and inhibited mast cell degranulation in mice with **oxaliplatin**-induced mechanical allodynia pain. The H_1 and H_4 receptors are likely molecular players in this process (Sakamoto, Andoh, & Kuraishi, 2016). For example, genetically silencing the H_4 receptor inhibited the production of IL-1β for human mast cells (Ebenezer, Prasad, Rajan, Thangam, & Transduction, 2018), and H_4 receptor activation was shown to stimulate a number of cytokines, including IL-6 (Jemima, Prema, & Thangam, 2014). In addition, while H_1 and H_2 receptor antagonism reduced hypersensitivity following nerve injury, it is possible that histamine released by mast cells contributes to the recruitment of neutrophils and macrophages in neuropathic pain and, acting via these histamine receptors, contributes to the regulation of hypersensitivity in this type of chronic pain (Jaggi et al., 2017; Smith et al., 2007; Zuo et al., 2003). There is an important aspect associated with H_2 receptor antagonism, which should be considered for its therapeutic potential in neuropathic pain control. In vitro studies using CHO and HEK-293 cells identified time- and dose-dependent up-regulation of H_2 receptors upon long-term exposure to H_2 receptor antagonists (e.g., ranitidine), which may underlie the development of tolerance after prolonged clinical use of these ligands and result in the rebound hypersecretion of gastric acid and anaphylaxis that can occur after withdrawal of treatment (Allen, Chazot, & Dixon, 2018; Smit et al., 1996). Thus, side effects linked to pharmacological tolerance may potentially compromise long-term efficacy and tolerability of H_2 receptor antagonists in neuropathic pain. Little is known about the role of the H_3 receptors in non-neuronal cells in neuropathic pain states.

Overall, non-neuronal cells play a key, but poorly, defined role in the mechanisms underlying histamine-mediated neuropathic pain. We propose that neuronal H_1 and H_4 receptors (Ferreira et al., 2012) may orchestrate these mechanisms, with IL-6 and IL-1β cytokines as common denominator mediators. Further complications arise from the recent observation that activated mast cells trigger microglial activation (Zhang, Wang, Dong, Xu, & Zhang, 2016). These cell types and their interactions may potentially go some way to explain the paradoxical effects of histamine ligands, particularly for the H_4 receptor, seen in animal pain models.

5 | HISTAMINE AND ITS INTERACTION WITH OPIOID SYSTEM IN NEUROPATHIC PAIN

Interestingly, in neuropathic pain, high doses of opioids are required to achieve pain relief, and pharmacological tolerance to analgesic effect of opioids develops rapidly (Osikowicz, Mika, Makuch, & Przewlocka, 2008). This phenomenon significantly restricts the clinical usefulness of opioids. In addition, the misuse of and addiction to

opioids, including prescription pain relievers, morphine and heroin, as well as synthetic opioids, such as fentanyl, is a serious international crisis that affects public health as well as social and economic welfare (Lipman & Webster, 2015).

An interaction between histaminergic and opioidergic systems within the CNS was suggested nearly 30 years ago, through an observation that morphine administration resulted in the release of histamine and its increased turnover in the periaqueductal grey (Nishibori, Oishi, Itoh, & Saeki, 1985), suggesting that analgesia produced by opioids may be associated with the stimulation of histamine receptors at the supraspinal level. There are also data suggesting that ligands of histamine receptors may modulate the analgesic action of opioids; however, the site and mode of this interaction differ between the spinal or supraspinal level, and depend on the subtype of histamine receptor involved (Mobarakeh et al., 2002; Mobarakeh et al., 2006; Mobarakeh, Takahashi, & Yanai, 2009). Specifically, a series of studies over the last two decades has shown that in H_1, H_2, or H_3 receptor-KO mice, morphine-induced antinociception was significantly augmented when compared to the wild-type controls in models of acute pain. H_1 receptor-KO mice showed a reduced spontaneous nociceptive threshold as they responded to significantly lower pain stimuli when compared to their controls (Mobarakeh et al., 2002), while thresholds for pain perception in H_2 receptor-KO mice were higher when compared to their corresponding controls (Mobarakeh et al., 2006). Intrathecal administration of morphine in H_1 and H_3 receptor-KO mice and i.c.v. morphine injection in H_2 receptor-KO mice produce enhanced analgesic effects (Mobarakeh et al., 2002; Mobarakeh et al., 2006). Interestingly, pharmacological blockade of H_1 and H_3 receptors by either intrathecal administration of the first-generation antihistamine chlorpheniramine (H_1 receptor antagonist) or thioperamide (H_3 receptor antagonist, H_3 /H_4 receptor inverse agonist), or H_2 receptor antagonism, produced by zolantidine (i.c.v. route), resulted in the potentiation of the morphine analgesic effect (Mobarakeh et al., 2002; Mobarakeh et al., 2006). These behavioural studies, in both KO mice and involving pharmacological interventions, clearly demonstrated that blocking H_1, H_2 and H_3 receptors in combination with morphine may have beneficial effects on analgesia and suggested that endogenous histamine may exert an inhibitory effect on morphine-induced analgesia acting via H_1 and H_3 receptors at the spinal cord level and via H_2 receptors at the supraspinal level.

Importantly, the observations observed with H_3 receptor-KO mice are consistent with a pharmacological study using a preclinical model of neuropathic pain induced by chronic constriction injury of the sciatic nerve. Here, Popiolek-Barczyk et al. (2018) showed that blockade of H_3 receptors by a selective antagonist (E-162) significantly enhanced morphine antinociception assessed with both mechanical and cold stimuli. Pharmacological analysis of these effects revealed an additive effect. Interestingly, Popiolek-Barczyk et al. (2018) also showed that TR-7, a selective H_4 receptor antagonist, significantly enhanced morphine antinociception in neuropathic pain. This latter study is the first demonstration of the involvement of H_4 receptors in the regulation of morphine efficacy in chronic pain.

To the best of our knowledge, the literature does not provide evidence for the mechanisms underlying histamine and opioid system interactions, in relation to the modulation of morphine analgesic effects. Given that the analgesic effects produced by modulation of the activity of both the histamine and opioid systems could be associated with blocking SP release from peripheral nerve terminals (Barnes et al., 1986; Przewłocki & Przewłocka, 2001), it is possible that an interaction that would result in potentiation of analgesic efficacy of morphine may involve, together with other possible mechanisms, the inhibition of peripheral SP accumulation. Such an outcome may be useful for the management of neuropathic pain, particularly when peripheral administration of drugs is possible, thus affording reduction of the undesired secondary effects associated with opioid administration and peripheral mechanisms of action (e.g., constipation). However, centrally acting drugs administered by peripheral routes should be taken into consideration due to the potential serious interactions related to their pharmacodynamics and central mechanisms of action. For example, chlorpheniramine (a first-generation H_1 receptor antagonist) was reported to potentiate fentanyl-induced sedation and respiratory depression after surgery (Anwari & Iqbal, 2003).

6 | CONCLUSIONS AND FUTURE DIRECTIONS

Findings from the last two decades indicate that selective pharmacological antagonism of neurons expressing H_3 receptors could provide important and promising therapeutic approaches for the control of mechanical and cold hypersensitivity in peripheral neuropathies (Table 1). The analgesic effectiveness of H_3 receptor antagonists/inverse agonists was comparable to gabapentin and pregabalin, first-line treatments for neuropathic pain. Importantly, multiple examples of behavioural, electrophysiological, and molecular evidence strongly support the rationale for this neuropathic pain strategy, particularly given their ability to modulate histamine levels as well as several neurotransmitters critical for chronic pain processing. Moreover, the recent registered approval of pitolisant (Wakix™), an antagonist/inverse agonist of H_3 receptors, for the treatment of narcolepsy in patients, has opened the door for the potential use of H_3 receptor ligands for other conditions, including chronic neuropathic pain. However, due to the wide presynaptic and postsynaptic distribution of H_3 receptors throughout the CNS and PNS, more research is certainly needed to clarify the involvement of peripheral, spinal, and brain H_3 receptors in various pain states, before determining their full potential in neuropathic pain.

Recent findings also suggest the use of centrally permeable H_2 receptor antagonists as promising new drug candidates for the treatment of neuropathic pain, in view of their analgesic effects and metabolic stability. Interestingly, however, despite the discovery of the most recently discovered histamine receptor, the role of the H_4 receptor in neuropathic pain transmission is still controversial after nearly 20 years, with apparent confounding effects of both agonists and antagonists on hypersensitivity associated with neuropathic pain. This

may be due to biased signalling of histamine and H_4 receptor agonist ligands and differential effects on multiple signalling pathways in central and peripheral parts of the sensory nervous system. Furthermore, the paucity of detailed mechanistic definitions of histamine-mediated analgesia, and the additive effects with the opioid system, requires attention to provide a rationale to the field of histamine and development of neuropathic pain control therapeutics.

A better understanding of the interaction between histaminergic signalling pathway molecules (Figures 1–3) and histamine receptors may result in the identification of further novel pharmacological targets to improve neuropathic pain control. The literature available provides some evidence for potential pharmacological target molecules. One potential strategy exploits the role of Ca^{2+} channels in the regulation of cellular excitability associated with nociception (e.g., N-type Ca^{2+} channels). Evidence has shown that Ca^{2+} channel blockers (e.g., **ω-conotoxin-MVIIA/Prialt**) offer interesting analgesic potential in treating neuropathic pain (Vanegas & Schaible, 2000). However, there is no evidence for the effect produced by a combination of Ca^{2+} channel blockers and histamine receptor ligands, and we propose that their interaction should be taken into consideration. Another potential target involves the contribution of the MAPK/ERK signalling pathway to the regulation of pain hypersensitivity. Recently, Sanna et al. (2015) showed that H_4 receptor stimulation, which led to analgesic activity in neuropathic pain, was modulated by MAPK/ERK signalling in the neurons of the DRG, spinal cord, and sciatic nerve. While the MAPK/ERK signalling pathway regulates pain sensitivity and, for a while, has been considered as a target for the treatment of neuropathic pain (Ma & Quirion, 2005), further studies on the interaction between this pathway and H_4 receptors may lead to the identification of more efficient therapeutic strategies to control neuropathic pain.

6.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org/, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017; Alexander, Peters et al., 2017; Alexander, Striessnig et al., 2017).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
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Votucalis, a Novel Centrally Sparing Histamine-Binding Protein, Attenuates Histaminergic Itch and Neuropathic Pain in Mice

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Votucalis is a biologically active protein in tick (*R. appendiculatus*) saliva, which specifically binds histamine with high affinity and, therefore, has the potential to inhibit the host's immunological responses at the feeding site. We hypothesized that scavenging of peripherally released endogenous histamine by Votucalis results in both anti-itch and anti-nociceptive effects. To test this hypothesis, adult male mice were subjected to histaminergic itch, as well as peripheral nerve injury that resulted in neuropathic pain. Thus, we selected models where peripherally released histamine was shown to be a key regulator. In these models, the animals received systemic (intraperitoneal, i.p.) or peripheral transdermal (subcutaneous, s.c. or intraplantar, i.pl.) administrations of Votucalis and itch behavior, as well as mechanical and thermal hypersensitivity, were evaluated. Selective histamine receptor antagonists were used to determine the involvement of histamine receptors in the effects produced by Votucalis. We also used the spontaneous object recognition test to confirm the centrally sparing properties of Votucalis. Our main finding shows that in histamine-dependent itch and neuropathic pain models peripheral (s.c. or i.pl.) administration of Votucalis displayed a longer duration of action for a lower dose range, when compared with Votucalis systemic (i.p.) effects. Stronger anti-itch effect was observed after co-administration of Votucalis (s.c.) and antagonists that inhibited peripheral histamine H_1 and H_2 receptors as well as central histamine H_4 receptors indicating the importance of these histamine receptors in itch. In neuropathic mice, Votucalis produced a potent and complete anti-nociceptive effect on mechanical hypersensitivity, while thermal (heat) hypersensitivity was largely unaffected. Overall, our findings further emphasize the key role for histamine in the regulation of histaminergic itch and chronic neuropathic pain. Given the effectiveness of Votucalis after peripheral transdermal administration, with a lack of central effects, we provide here the first evidence that scavenging of peripherally released histamine by Votucalis may represent a novel therapeutically effective and safe long-term strategy for the management of these refractory health conditions.

Keywords: Votucalis, histamine-binding protein, histamine receptor, itch, neuropathic pain, CNS-sparing, mice

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INTRODUCTION

Histamine [2-(4-imidazolyl)-ethylamine], that can be found in almost all tissues of the mammalian body (Haas et al., 2008), is synthesised and stored primarily in cytosolic granules of the peripheral and central tissues, mast cells, basophils, eosinophils, platelets, basophiles, histaminergic neurons and enterochromaffin cells (Benly, 2015; Branco et al., 2018). Also, other types of non-professional histamine cells, such as epithelial cells, dendritic cells, neutrophils and T lymphocytes can synthesize and secrete histamine immediately after its production, although they do not store it (O'Mahony et al., 2011). Given its wide distribution in multiple cell types, histamine has been shown to regulate many physiologic and pathologic conditions, including pruritus/itch (Baron et al., 2001) and chronic pain (Obara et al., 2020). Histamine produces these regulatory effects *via* four G protein-coupled histamine receptors: H_1, H_2, H_3 and H_4 receptors that are expressed in both the central and peripheral nervous system (CNS and PNS, respectively) (Lindskog, 2017). Apart from distribution, histamine receptors differ in their pharmacological and signal transduction properties, and for that reason histamine has been shown to have different effects depending on the histamine receptor subtype it is bound to (Ikoma et al., 2006; Panula et al., 2015; Lindskog, 2017; Obara et al., 2020). For example, histamine depending on its concentration, site of action and type of receptors implicated, can both reduce and aggravate sensory perception of itch and pain (Tamaddonfard and Rahimi, 2004; Ikoma et al., 2006). Therefore, histamine as well as histamine receptors have long been attractive targets for therapeutic interventions in conditions where itch and pain are symptoms requiring treatment (O'Donoghue and Tharp, 2005; Obara et al., 2020). However, despite these multiple efforts, the understanding of itch signalling *via* histamine system is far from completely understood (Rossbach et al., 2011), and standard antihistamine treatments targeting H_1 receptors showed limited efficacy (Patel and Yosipovitch, 2010). Also, findings reporting roles played by histamine in chronic pain are somewhat contradictory (Haas et al., 2008; Obara et al., 2020). Thus, the therapeutic potential of histamine modulation still requires clarification.

The discovery of histamine-binding proteins in the saliva of arthropods/ticks has provided a new and powerful tool to revisit the role of histamine in the regulation of itch and pain (Weston-Davies et al., 2005; Chmelař et al., 2019). Ticks are species of blood-feeding arthropods with animal and human hosts and have adopted a specialized strategy to suppress host-defense immunological mechanisms at the feeding site (Paesen et al., 1999; Ryffel et al., 2005). The saliva of the tick contains complex bioactive molecules, including a selection of proteins and lipids with anti-inflammatory, anti-coagulant, anti-platelet, anti-fibrotic, anti-hemostatic and immunomodulatory effects (Štibrániová et al., 2019). Despite the identification of a large number of highly bioactive tick salivary molecules, their development for medical purposes remains in its infancy. Studies have been performed on only a small number of tick salivary compounds, mainly proteins (Aounallah et al., 2020). Votucalis (also known as EV131 or HPB1), one of the proteins extracted from female *Rhipicephalus Appendiculatus* tick displays histamine binding properties and is linked to a broad ligand-binding protein family, known as lipocalins. Votucalis is a histacalin, which captures histamine within two different internal binding sites with the high-affinity binding site displaying a 100-fold higher affinity than H_1 and H_2 receptors, and a similar affinity to the H_3 and H_4 receptors (Paesen et al., 1999; Paesen et al., 2000). Interestingly, there are both pre-clinical and clinical studies showing therapeutic effects achieved by scavenging of endogenous histamine by recombinant Votucalis. These studies focused on conditions where histamine is recognized as an important inflammatory mediator and included models of acute respiratory distress syndrome (ARDS), asthma and allergic rhinitis (Ryffel et al., 2005).

This study further explored novel refractory therapeutic indications based on scavenging of peripherally released endogenous histamine by Votucalis and focused on mouse models of acute itch and chronic neuropathic pain. Histamine is a known key regulator of both conditions and we, therefore, hypothesized that selective targeting of peripheral histamine by Votucalis would result in both anti-pruritic and anti-nociceptive effects that may suggest novel and safe therapeutic approaches in the regulation and control of these conditions.

MATERIALS AND METHODS

Subjects

C57BL6/J mice. Experimental protocols (PPL: P8E3496FA, P6694C943) were performed under UK Home Office license, with the Animal Welfare Ethical Review Body (AWERB) local approval, and in accordance with current UK legislation as defined in the Animals (Scientific Procedures) Act 1986. The Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) has been followed in reporting this study.

Adult male C57BL/6J mice (8 weeks of age; 20–25 g; Charles River Laboratories, Kent, UK) were allowed to acclimatize to the colony room (Life Sciences Support Unit Durham University, UK and Comparative Biology Centre, Newcastle University, UK) for at least 7 days after arrival, and were housed in polyethylene cages (4 per cage), controlled for temperature (21°C) and humidity (55%) under a regular 12-h day/night cycle (lights on at 8:00 a.m.; lights off at 8:00 p.m.). Standard laboratory rodent chow and water were available *ad libitum*. Animals were habituated to testing procedures for at least 3–4 days before experiments. The handling and testing of the animals were conducted during the light phase, between 9:00 a.m. and 4:00 p.m. All efforts were made to minimize animal suffering and to reduce the number of animals used in the study.

Material: Preparation and Administration

Votucalis was provided by Akari Therapeutics Plc (UK) as a stock solution of 5.8 mg ml^{-1} in phosphate-buffered saline and was stored at −80°C (histamine binding (K_D) to Votucalis = 1.6 nM, data not shown). For all administrations, Votucalis was thawed immediately before injections and prepared in a vehicle (sterile

saline; 0.9% NaCl; Fresenius Kabi Ltd., UK) solution at required concentrations, as described below. The isolation, cloning and detailed crystal structure of Votucalis were reported previously (Paesen et al., 1999; Paesen et al., 2000).

Systemic i.p. administration. Mice were weighed and randomised to receive either Votucalis or vehicle; they were injected i.p. with Votucalis at 1, 3, 10, 20 and 40 mg kg^{-1} body weight or vehicle (saline) solution as a control group. In the itch model, Votucalis/vehicle was injected 30 min before injection of pruritogens. In the neuropathic pain model, Votucalis/vehicle was administered once every 24 h on days 7, 8, 9 and 10 post induction of neuropathic pain.

Peripheral s.c. administration. Mice were weighed and randomised to receive either Votucalis or vehicle; they were injected s.c. with Votucalis at 0.3, 1, 3, 10 and 20 mg kg^{-1} body weight or vehicle (saline) solution as a control group. In the itch model, Votucalis/vehicle was injected once 30 min before injection of pruritogens. In the spontaneous object recognition task performed in naïve animals, Votucalis (20 mg kg^{-1} body weight) was injected once 30 min before the task, and then the injection was repeated every 24 h for a total of 4 days.

Peripheral intraplantar (i.pl.) administration. Mice were randomized to receive either Votucalis or vehicle; they were injected i.pl. with Votucalis (0.0075, 0.025, 0.075 and 0.25 mg paw^{-1}) or equivalent vehicle (saline) solution without Votucalis as a control group. Injections were given over 1 min in a volume of 50 µL without anesthesia into the plantar surface of the animal hind paw ipsilateral to sciatic nerve injury. In the neuropathic pain model, Votucalis/vehicle was administered once every 24 h on days 7, 8, 9 and 10 post induction of neuropathic pain.

To determine the involvement of histamine receptors in the effects produced by Votucalis as well as to potentiate the anti-pruritic and anti-nociceptive effects produced by Votucalis, selective H_1 receptor antagonist (mepyramine maleate, 10 and 20 mg kg^{-1}; Sigma-Aldrich, UK), selective H_2 receptor antagonist (ranitidine hydrochloride, 15 mg kg^{-1}; Tocris Bioscience, UK) and selective H_4 receptor antagonist (JNJ 7777120, 20 mg kg^{-1}; Tocris Bioscience, UK) were co-injected with Votucalis or alone 30 min before injection of pruritogen. The antagonists were dissolved immediately before injections (i.p. or s.c.) in sterile saline (0.9% NaCl; Fresenius Kabi Ltd., UK), except JNJ 7777120 which was dissolved in DMSO (dimethyl sulfoxide, 5%; Sigma-Aldrich, UK). Control animals received equivalent vehicle (saline or 5% DMSO) injections. The concentration and timing of antagonist injections and measurements were based on previously published reports (Bell et al., 2004; Dunford et al., 2007; Rossbach et al., 2011).

Itch Model

Induction of itch. As previously published (Sun et al., 2009; Obara et al., 2015), histamine-dependent itch was induced in mice by injection of compound 48/80 (100 µg; Sigma-Aldrich, UK), while histamine-independent itch was induced by injection of chloroquine (chloroquine diphosphate salt, 200 µg; Sigma-Aldrich, UK). Itch-inducing agents were dissolved in sterile saline and administered s.c. into the nape of the mouse neck in a volume of 50 µL. Prior to itch experiments, the back of the mouse neck was shaved, and animals were given 30 min to acclimatize to a small plastic chamber. For administration of pruritogens, mice were removed from the chamber, gently restrained, and injected. Itch behavior in the mouse that developed after s.c. injection of pruritogens was recorded with a digital video camera and analyzed.

Behavioral assessment. In all experiments, the observer was not aware of the doses and/or treatment administered. One scratch was defined as a lifting of the hind limb toward the injection site (the shaved area of the neck) and then replacing the limb back to the floor, regardless of how many scratching strokes (bouts of scratching) took place between those two movements (Shimada et al., 2006; Obara et al., 2011). The scratching was quantified as the total number of scratches across a 40-min observation period, or the cumulative number of scratches observed for 40 min at 5-min intervals. Each mouse was used only once, in one experiment.

Neuropathic Pain Model

Induction of neuropathic pain. As previously published (Bennett and Xie, 1988; Obara et al., 2013), mice were subjected to peripheral neuropathy induced by chronic constriction injury (CCI) of the sciatic nerve. The injury was performed under isoflurane anesthesia delivered *via* a nose cone (up to 5% isoflurane with oxygen as the carrier gas for induction and 1.5–2.5% for maintenance). The skin on the lateral surface of the left thigh was shaved and an incision was made just below the left hipbone, parallel to the sciatic nerve. The *biceps femoris* and the *gluteus superficialis* were separated and the left sciatic nerve was exposed. Proximal to the sciatic trifurcation, the injury was produced by three loose ligations (4/0 silk) around the sciatic nerve. The ligatures were tied loosely around the nerve with 1 mm spacing, until they elicit a brief twitch in the respective hindlimb, which prevented the application of an excessively strong ligation. The total length of nerve affected was 3–4 mm. After the ligation, the muscle and skin were closed in two separate layers. In sham control mice, the sciatic nerve was exposed as described above but no contact was made with the nerve.

Behavioral assessment. In all experiments, the observer was not aware of the dose of Votucalis administered.

Mechanical hypersensitivity was assessed by measuring the withdrawal threshold of the mouse paw in response to mechanical stimuli using von Frey filaments in the 0.04–6.0 g range (Stoelting, Wood Dale, IL, USA). Mice were placed individually in a plastic cage (10 × 6 × 6 cm) with a metal mesh floor and were allowed to habituate before testing began. Animals were also habituated over a period of 3–4 consecutive days by recording a series of baseline measurements. The filaments were applied in ascending order of strength, each five times at an interval of 2–3 s to the plantar surface of the hind paw as described previously (Bourquin et al., 2006; Obara et al., 2011). The smallest filament eliciting a foot withdrawal response was considered the threshold stimulus. Data were collected from both the ipsilateral and contralateral paw to the side of the injury.

Thermal (heat) hypersensitivity (Hargreaves test) was assessed by measuring the latency of paw withdrawal response to a

noxious thermal stimulus using a radiant heat-emitting device (IITC Life Science Inc., USA), as described previously (Hargreaves et al., 1988; Obara et al., 2011). Mice were placed individually in a plastic cage (12 × 10 × 10 cm) on an elevated glass platform and allowed to habituate to the apparatus before testing began. Animals were also habituated over a period of 3–4 consecutive days by recording a series of baseline measurements. A radiant heat source of constant intensity was applied to the plantar surface of the paw through the glass plate and the latency to paw withdrawal was measured. The hind paw received three stimuli and the inter-stimulus interval was at least 3–5 min to prevent injury. Withdrawal latencies were defined as the mean of the three readings for each hind paw. A cut-off of 20 s was employed to avoid tissue injury. Data was collected from both the ipsilateral and contralateral paw to the side of the injury.

Both mechanical hypersensitivity at the lateral plantar surface of the hind paw and heat hypersensitivity were assessed before nerve injury (as basal pain threshold) and then testing commenced on day 7 after the sciatic nerve injury and continued for three consecutive days with behavioral testing on day 7, 8, 9 and 10 post injury. Both tests were performed prior to administration of Votucalis (i.p. or i.pl.) and were repeated at 30 min, 1, 2, 4, 6, 8 and 24 h after each Votucalis administration on the four test days. Each animal first underwent von Frey testing followed by the Hargreaves test.

Spontaneous Object Recognition Task

Short-term recognition memory was assessed using the spontaneous object recognition (SOR) task (Ennaceur and Delacour, 1987).

Behavioral assessment. In all experiments, the observer was not aware of the doses and/or treatment administered. The animals received a total of four 12-min habituation sessions. During the first 3 days of habituation, mice were placed individually in a matte black wooden box (60 × 60 × 60 cm) and allowed to explore the apparatus for 12 min. On the 4th day of habituation, mice were exposed to an object placed in the middle of the apparatus. The animals did not re-encounter this object afterwards, during the object recognition test.

The task consisted of an exposure and test phase, each lasting 3 min. During the exposure phase, mice were exposed to an identical pair of objects (A_1 and A_2), placed at the back-left and back-right corner of the wall, about 4 cm from the wall. Following a 1-h delay, mice were placed back into the apparatus to explore a copy of the familiar object (A_3) and a novel object (B). Each object was available in triplicates and the objects were cleaned with 3% hydrogen peroxide solution (EndoSan, UK) between animals to avoid unwanted olfactory cues. The location and order in which the novel objects presented were counterbalanced. Exploratory behavior was recorded by an overhead video camera on one side of the apparatus. The animals were tested for recognition memory on the 1st and 4th day of treatment with Votucalis (s.c.). The effects of Votucalis were compared to mepyramine maleate (10 mg kg^{-1} body weight, i.p.) and vehicle (saline, i.p.).

Object exploration was defined as the nose of the mouse being directed towards the object at less than 1 cm. Novelty preference was measured by dividing the difference score (difference score: novel minus familiar) with the total exploration time. This results in the discrimination ratio ranging between -1 and 1, with a negative score indicating a preference to the familiar object and a positive score indicating a preference for the novel object (Ennaceur and Delacour, 1988).

Data and Statistical Analysis

All data were recorded with Microsoft Excel. Itch data were collected as videos. Mice were randomly assigned to experimental groups. Each group included 5–18 mice, however the exact group size for each experimental group/condition is indicated in the figure legend for each figure. Data analysis and statistical comparisons were performed using GraphPad PrismTM, version 7.00 for Windows/OS (GraphPad Software, CA, USA, www.graphpad.com). Statistical analysis was performed by one- or two-way ANOVA with Bonferroni's multiple comparison post-hoc tests or by unpaired Student's t-test when two groups were compared. A value of $p < 0.05$ was considered to be statistically significant. Results are presented as mean ± standard error of the mean (SEM).

RESULTS

Systemic Administration of Votucalis Reduced Histaminergic, but Not Non-histaminergic, Itch Behavior

Administration (s.c.) of both compound 48/80 and chloroquine induced scratching behaviour that lasted for ~40 min (**Figures 1A,B** respectively; total number of scratches in the vehicle control group for compound 48/80: 283.9 ± 23.4 and for chloroquine: 461.2 ± 30.2). Dose-dependent significant inhibition of itch behaviour resulting from systemic (i.p.) treatment with Votucalis was only observed in histamine-dependent itch induced by compound 48/80 (**Figure 1A**; drug effect: $F_{(5,339)} = 6.4$, $p < 0.0001$). The anti-itch effect produced by Votucalis was not observed for the whole 40 min of the observation period, when compared with vehicle control animals ($p > 0.05$). As indicated in **Figure 1C**, systemic Votucalis promoted significant inhibition of histamine-dependent itch within the first 20 min from the induction of itch. The effect was observed only for the two highest doses of Votucalis (20 and 40 mg kg^{-1}), while lower doses of Votucalis (1, 3 and 10 mg kg^{-1}) did not show any significant differences between treated and saline control groups ($F_{(5,41)} = 3.5$, $p = 0.01$). Votucalis did not significantly reduce scratching behaviour caused by chloroquine, showing its ineffectiveness for histamine-independent itch (**Figures 1B,D**; $p > 0.05$).

Peripheral Administration of Votucalis Reduced Histaminergic Itch Behavior

Peripheral (s.c.) administration of Votucalis inhibited histamine-dependent itch behaviour induced by compound 48/80 (**Figure 2A**; drug effect: $F_{(5,328)} = 50.43$, $p < 0.0001$). This effect was observed over a 40-min observation period in mice

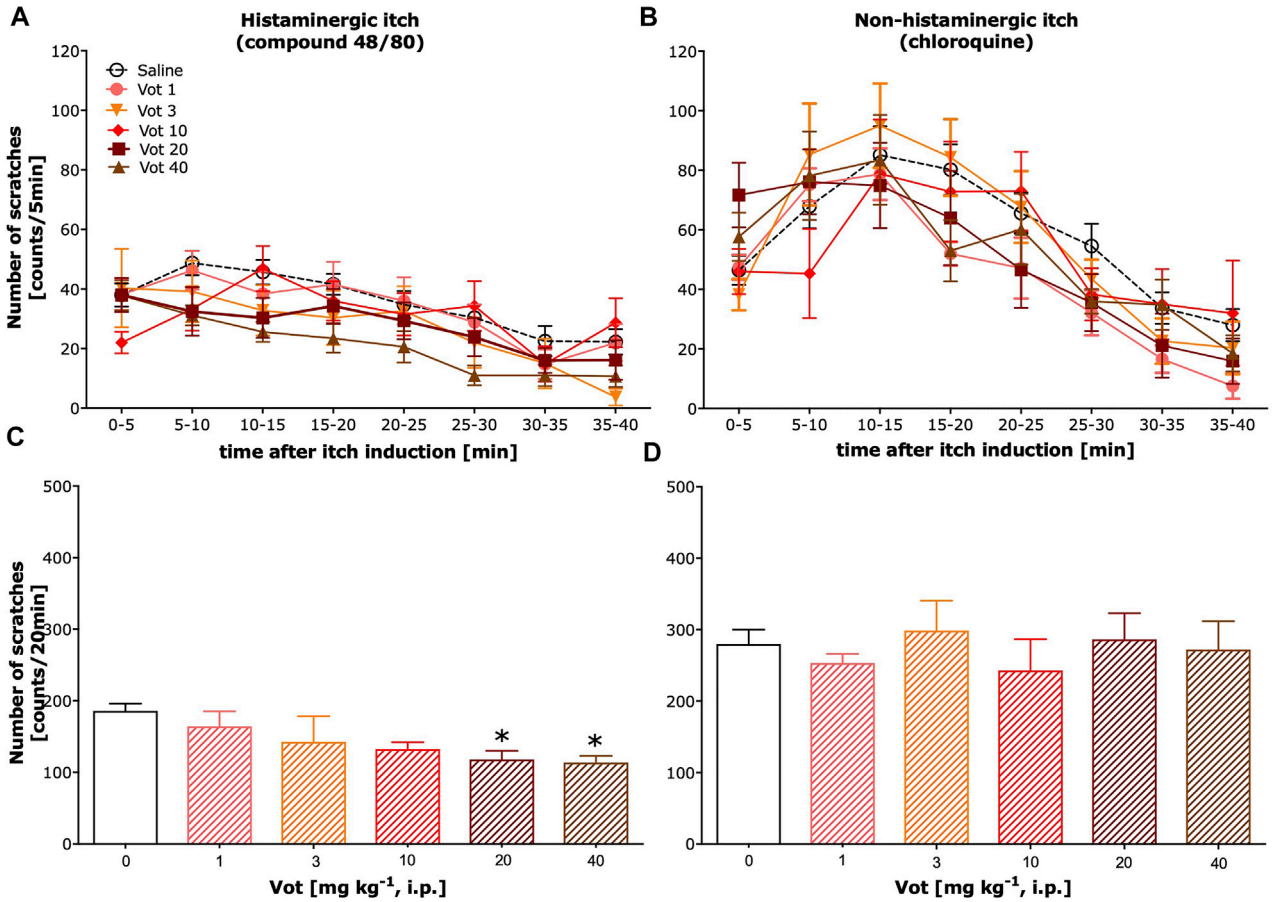

FIGURE 1 | Systemic administration of Votucalis inhibited itch behaviour induced by histamine-dependent, but not histamine-independent pruritogens, in mice. **(A,B)** Time-course effect of systemic (i.p.) injection of Votucalis (Vot), or vehicle (saline), on the total number of scratches that occurred over a 40-min time period after injection of compound 48/80 **(A)** or chloroquine **(B)** into the nape of the mouse neck. Mice received an injection of Votucalis (1–40 mg kg^{-1}; n = 6–9) or vehicle (saline; n = 14–18) 30 min before the injection of pruritic agents. Itch behaviour was recorded, and scratches were counted in 5-min intervals for 40 min **(C,D)** Bar graphs displaying the total number of scratches across the first 20-min observation period after injection of compound 48/80 **(C)** or chloroquine **(D)** for each treatment. Data are presented as means ± SEM; the asterisk (*) denotes significance vs. vehicle control animals; *$p < 0.05$ (one-way ANOVA, followed by Bonferroni's comparison post-hoc test).

indicating a longer duration of action in comparison with the systemic (i.p.) effect produced by Votucalis (**Figure 2B**; $F_{(5,41)}$ = 14.6, $p < 0.0001$). In addition, the anti-itch effect produced by Votucalis was dose-dependent for the dose range between 0.3 and 10 mg/kg (**Figure 2B**). However, the highest tested dose of 20 mg kg^{-1} showed decreased anti-itch effect compared to the lower doses of Votucalis tested, although the effect was still significant when compared with the saline control (**Figure 2B**; p = 0.003). This observation indicates a biphasic or bell-shape dose-response curve produced by peripherally administered Votucalis.

Peripheral Histamine H_1 and H_2 Receptors as Well as Central Histamine H_4 Receptors Potentiated the Effects of Votucalis

As illustrated in **Figures 3A,B**, mepyramine, a selective H_1 receptor antagonist, when injected systemically (i.p.) required a high dose to reduce histamine-dependent itch produced by compound 48/80 as 10 mg kg^{-1} was ineffective ($p > 0.05$), while 20 mg kg^{-1} significantly inhibited compound 48/80-induced itch ($p < 0.0001$). In contrast, peripheral (s.c.) administration of mepyramine at the dose of 10 mg kg^{-1} was significantly effective in inhibiting of compound 48/80-induced itch ($p < 0.0001$). This peripherally effective dose of mepyramine when co-administered with Votucalis (10 mg kg^{-1}, s.c.) produced stronger inhibition of histamine-dependent itch induced by compound 48/80 (p = 0.007) when compared to the effect produced by the drugs alone. Systemic administration of mepyramine (20 mg kg^{-1}, i.p.) together with Votucalis (10 mg kg^{-1}, s.c.) did not attenuate the anti-itch effect of the drugs alone ($p > 0.05$).

As illustrated in **Figures 3C,D**, ranitidine, a selective H_2 receptor antagonist, when injected systemically (i.p.) at the dose of 15 mg kg^{-1} did not reduce histamine-dependent itch produced by compound 48/80 ($p > 0.05$). The same dose of

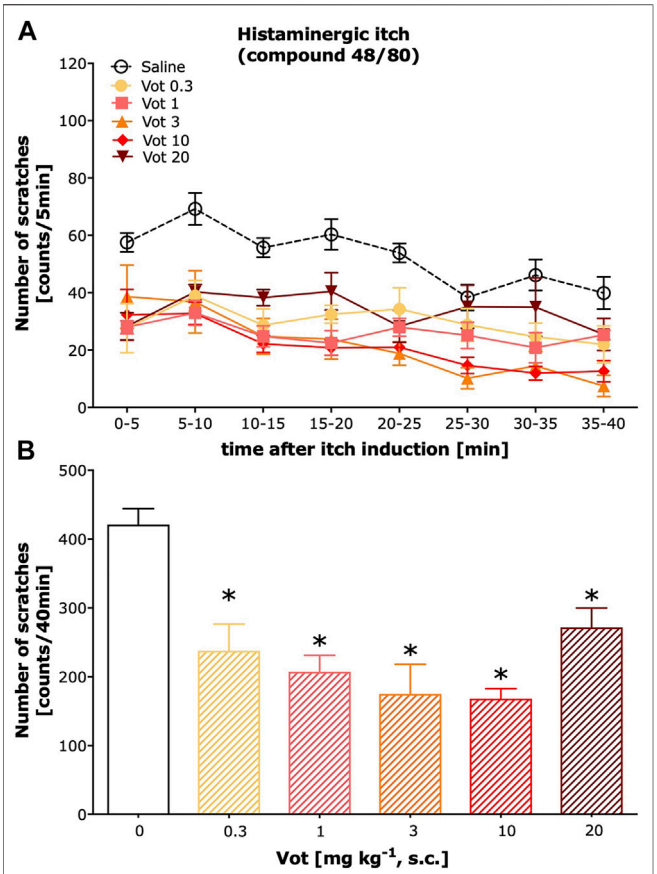

FIGURE 2 | Peripheral administration of Votucalis inhibited itch behaviour induced by histamine-dependent pruritogen in mice. **(A)** Time-course effect of peripheral (s.c.) injection of Votucalis (Vot), or vehicle (saline), on the total number of scratches that occurred over a 40-min time period after injection of compound 48/80 into the nape of the mouse neck. Mice received an injection of Votucalis (0.3–20 mg kg^{-1}; $n = 6$) or vehicle (saline; $n = 17$) 30 min before the injection of compound 48/80. Itch behaviour was recorded, and scratches were counted in 5-min intervals for 40 min. **(B)** Bar graph displaying a total number of scratches across a 40-min observation period for each treatment. Data are presented as means ± SEM; the asterisk (*) denotes significance vs. vehicle control animals; *$p < 0.05$ (one-way ANOVA, followed by Bonferroni's comparison post-hoc test).

ranitidine when injected peripherally (s.c.) significantly reduced compound 48/80-induced itch ($p < 0.0001$), as well as the anti-itch effect produced by peripherally co-administered Votucalis (10 mg kg^{-1}, s.c.) and ranitidine (15 mg kg^{1-}, s.c.) was stronger when compared to the effect produced by the drugs alone ($p = 0.002$). Systemic administration of ranitidine (15 mg kg^{1-}, i.p.) together with Votucalis (10 mg kg^{-1}, s.c.) did not attenuated the anti-itch effect when compared to the effect produced by the drugs alone ($p > 0.05$).

As illustrated in **Figures 3E,F**, JNJ 7777120, a selective H_4 receptor antagonist, when injected either systemically (i.p.) or peripherally (s.c.) at the dose of 20 mg kg^{-1} significantly reduced histamine-dependent itch produced by compound 48/80 ($p < 0.0001$ and $p = 0.0004$ respectively). However, only systemically administered JNJ 7777120 (20 mg kg^{-1}, i.p.) when co-administered with Votucalis (10 mg kg^{-1}, s.c.) produced stronger inhibition of compound 48/80-induced itch ($p = 0.002$), while peripheral administration of JNJ 7777120 (20 mg kg^{-1}, s.c.) did not attenuate the anti-itch effect when co-administered with Votucalis (10 mg kg^{-1}, s.c.; $p > 0.05$).

In summary, Votucalis provided inhibition of histaminergic itch around 62%, which was further increased by both peripheral H_1 and H_2 or central H_4 antagonism up to around 85, 90 and 81%, respectively. Peripheral H_1 and H_2 or central H_4 antagonism without Votucails inhibited histaminergic itch around 64, 53 and 27%, respectively. The incomplete inhibition of itch by Votucalis is assumed to be due to high levels of histamine released by mast cell degranulation in this model.

Systemic and Peripheral Administration of Votucalis Reduced Mechanical Hypersensitivity

The analgesic effectiveness of systemic (i.p.) administration of Votucalis was assessed by measuring the paw withdrawal threshold in response to mechanical stimuli using von Frey filaments in mice with neuropathic pain (CCI model). After systemic administration of Votucalis, the mechanical hypersensitivity in CCI mice was significantly reduced (**Figure 4A**; drug effect: F (5,812) = 300.8, $p < 0.0001$) in a dose dependent manner. The three highest doses of Votucalis, 10, 20 and 40 mg kg^{-1}, significantly attenuated mechanical hypersensitivity compared to the saline control (**Figure 4B**; $F_{(5,32)} = 102.2$, $p < 0.0001$). The greatest reduction in mechanical hypersensitivity was observed 1 and 2 h after the first injection of systemic Votucalis (10, 20 and 40 mg kg^{-1}), when compared with saline controls, and this effect was maintained at 4 h after the administration, but 24 h after the administration, was no longer observed (**Figure 4A**). A similar pattern of analgesic effect was observed after each of four consecutives daily systemic administration of Votucalis (10, 20 and 40 mg kg^{-1}; **Figure 4A**), indicating lack of pharmacological tolerance. No significant difference in mechanical hypersensitivity was seen with the lowest doses of Votucalis, 1 and 3 mg kg^{-1}, when compared to the saline control (**Figures 4A,B**; $p > 0.05$).

The analgesic efficacy of peripheral (i.pl.) administration of Votucalis was assessed by measuring the paw withdrawal threshold in response to mechanical stimulus using von Frey filaments in CCI mice. After peripheral administration of Votucalis, the mechanical hypersensitivity in CCI mice was significantly reduced with the three highest doses 0.025, 0.075 and 0.25 mg paw^{-1} when compared to vehicle control (**Figures 4C,D**; $F_{(4,25)} = 93.38$, $p < 0.0001$). The greatest reduction in mechanical hypersensitivity was observed 4 h after the first injection of Votucalis (0.025, 0.075 and 0.25 mg paw^{-1}), when compared with saline controls and this effect gradually declined, and 24 h after the administration was no longer observed (**Figure 4C**). A similar pattern of analgesic effect was observed after each of the four consecutive daily i.pl. administrations of Votucalis (0.025, 0.075 and 0.25 mg paw^{-1}; **Figure 4C**), indicating a lack of pharmacological tolerance. No significant difference in mechanical hypersensitivity was seen with the lowest dose of Votucalis, 0.0075 mg paw^{-1}, when compared to the saline control (**Figures 4C,D**; $p > 0.05$).

Sham mice, after systemic (i.p.) or peripheral (i.pl.) administration of Votucalis (40 mg kg^{-1} or 0.25 mg paw^{-1})

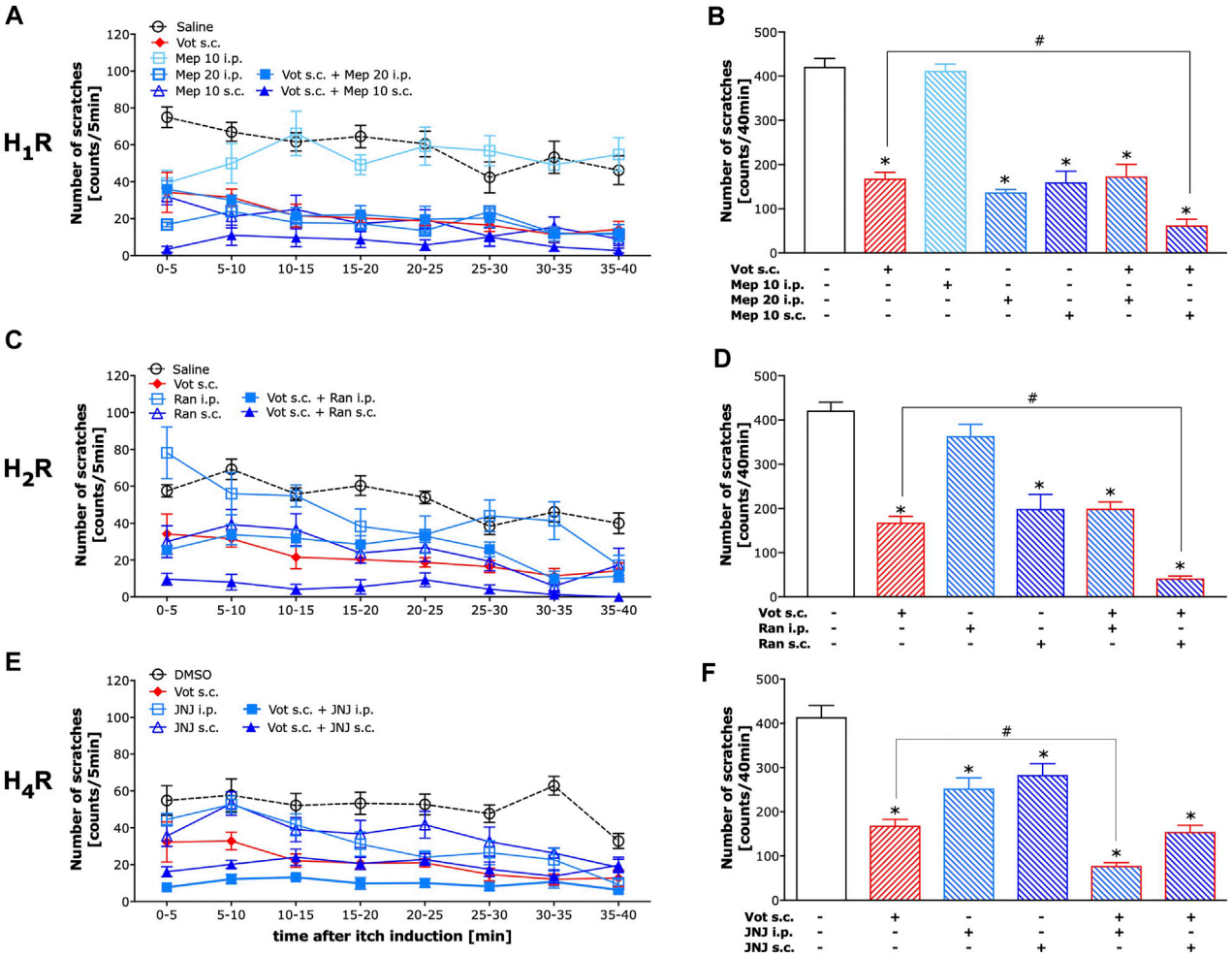

FIGURE 3 | Peripheral H_1 and H_2 receptors, as well as central H_4 receptors, attenuated the anti-itch effect induced by Votucalis in mice. Left panel **(A,C,E)** Time-course effect of injection of Votucalis (Vot, 10 mg kg^{-1}; s.c.; $n = 9$; A, C, E), H_1 receptor (H_1R) antagonist mepyramine (Mep, 10–20 mg kg^{-1}; i.p. or s.c.; $n = 6$; A), H_2 receptor (H_2R) antagonist ranitidine (Ran, 15 mg kg^{-1}; i.p. or s.c.; $n = 6$; B), H_4 receptor (H_4R) antagonist JNJ 7777120 (JNJ, 20 mg kg^{-1}; i.p. or s.c.; $n = 6$; C) or vehicle (saline or DMSO, $n = 6$–17, **A,C,E**), on the total number of scratches that occurred over a 40-min time period after injection of compound 48/80 into the nape of the mouse neck. Mice received a single injection of each of the drugs or a combination of Votucalis with one of the histamine receptor antagonists or vehicle 30 min before the injection of pruritic agent. Itch behaviour was recorded, and scratches were counted in 5-min intervals for 40 min. Right panel **(B,D,F)** Bar graphs displaying a total number of scratches across the 40-min observation period for each treatment. Data are presented as means ± SEM, $n = 6$–17 in each group. The drugs administered to each group is indicated below the respective bar. The asterisk (*) denotes significance *vs.* vehicle control animals; *$p < 0.05$ (one-way ANOVA, followed by Bonferroni's comparison post-hoc test). The hash (#) denotes significance vs. Votucalis treated animals; #$p < 0.05$ (one-way ANOVA, followed by Bonferroni's comparison post-hoc test).

showed a lack of change in paw withdrawal threshold (**Figures 6A,B**; $p > 0.05$). Only a weak significant effect was observed after localised peripheral administration of Votucalis into the ipsilateral paw (to CCI), when the analgesic effect was assessed on the contralateral paw (**Figure 6C**; $t_{(11)} = 3.4$, $p = 0.006$).

Systemic, but Not Peripheral, Administration of Votucalis Produced a Weak Effect on Neuropathic Pain-Induced Thermal (Heat) Hypersensitivity

The analgesic efficacy of systemic (i.p.) and peripheral (i.pl.) administration of Votucalis was also assessed by measuring the paw withdrawal latency in response to heat stimuli using the Hargreaves test in CCI mice. Systemic administration of Votucalis produced a weak effect on heat hypersensitivity (**Figures 5A,B**; $F_{(5,32)} = 2.8$, $p = 0.03$, Bonferroni's comparison post-hoc test non-significant). However, no significant difference in heat hypersensitivity was observed at any doses of Votucalis tested after peripheral administration (**Figures 5C,D**; $F_{(4,25)} = 1.2$, $p = 0.34$).

No change in paw withdrawal latency to heat stimuli was observed in sham mice after either systemic or peripheral (i.pl.) administration of Votucalis (40 mg kg^{-1} or 0.25 mg paw^{-1}) in ipsilateral or contralateral paws (**Figures 6D–F**; $p > 0.05$).

Votucalis, in any of the tested doses administered, either systemically or peripherally, did not cause any form of visible

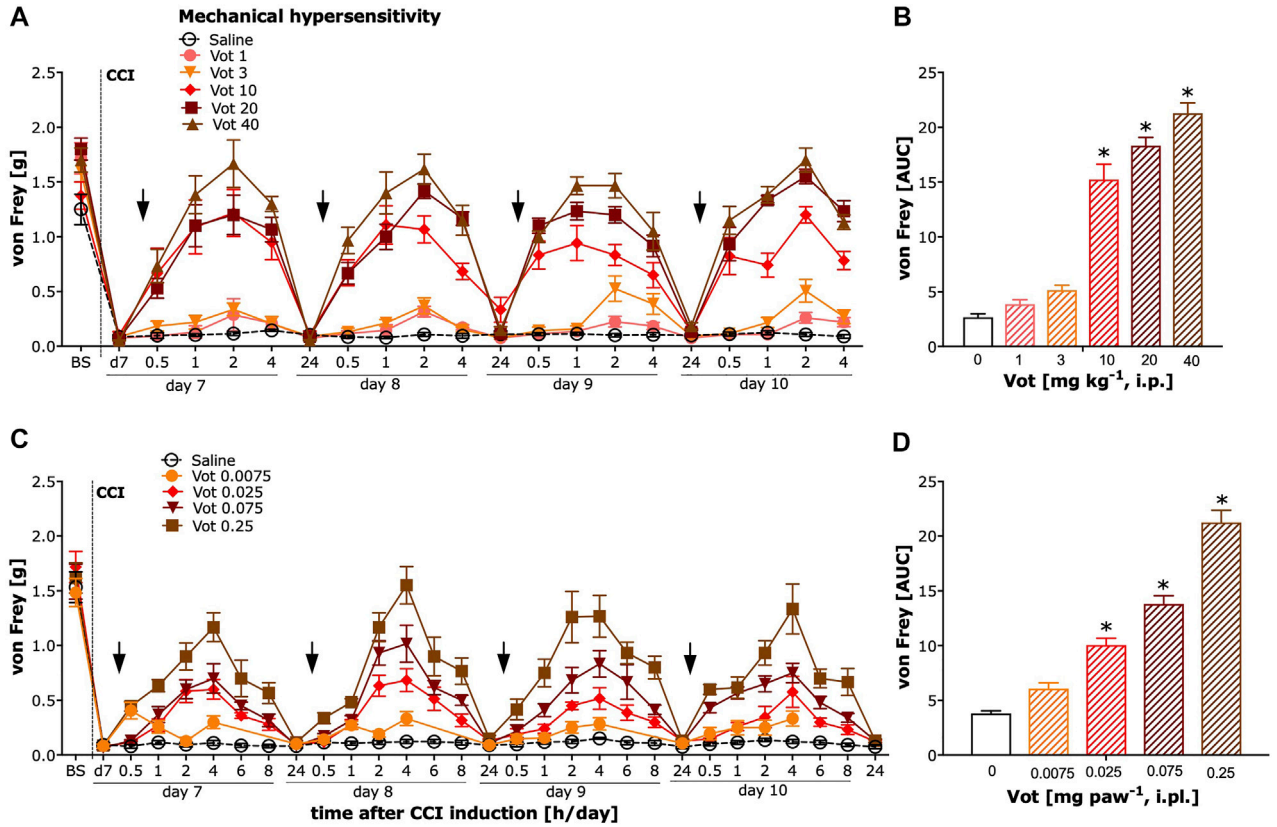

FIGURE 4 | Chronic systemic and peripheral administration of Votucalis significantly attenuated mechanical hypersensitivity in the chronic constriction injury (CCI) model of neuropathic pain in mice. Left panel **(A,C)** Time-course effect of systemic (i.p.) injection of Votucalis (Vot, 1–40 mg kg^{-1}, $n = 6$; A) and peripheral (i.pl.) injection of Votucalis (Vot, 0.0075–0.25 mg paw^{-1}, n = 6–12; C) administered into the plantar surface of the ipsilateral paw to injury, or vehicle (saline, n = 6–14; A, C), on the mechanical withdrawal threshold measured with von Frey filaments. The measurements were assessed before injury as basal pain threshold (BS), and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5–24 h after each of four consecutive once daily (arrow) intraperitoneal **(A)** or intraplantar **(C)** injections. Data are presented as means ± SEM, $n = 6$–14 in each group. Right panel **(B,D) (B)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in C. The asterisk (*) denotes significance vs. vehicle control animals; *$p < 0.05$ (one-way ANOVA, followed by Bonferroni's comparison post-hoc test).

discomfort throughout the period of 4 days, and there were no significant changes in mouse body weights during this study (data not shown).

Peripheral Administration of Votucalis Produced Anti-pruritic and Anti-nociceptive Effects at a Lower Dose Range Compared to Systemic Administration

As summarized in **Figure 7**, Votucalis produced dose-dependent anti-itch effects on histamine-dependent itch produced by compound 48/80 (**Figure 7A**), and anti-nociceptive effects on mechanical hypersensitivity resulting from injury of the sciatic nerve and subsequent development of neuropathic pain (**Figure 7B**). Overall, peripheral (s.c. or i.pl.) administration of Votucalis produced anti-itch and anti-nociceptive effects at a lower dose range in comparison to systemic (i.p.) administration indicating higher potency after peripheral transdermal Votucalis administrations observed as a left-ward shift of the dose-response curve for Votucalis.

Peripheral Administration of Votucalis did Not Affect Short-Term Recognition Memory

Single peripheral (s.c.) administration of Votucalis preserved short-term recognition memory in naïve mice while systemic (i.p.) administration of H_1 receptor antagonist mepyramine resulted in a significant impairment in short-term recognition memory. Similar effect was also observed when Votucalis and mepyramine were administered across four consecutive days (**Figures 8A,B**; drug effect: $F_{(2,21)} = 12.2$ $p = 0.0003$ and $F_{(2,21)} = 10.05$ $p = 0.0009$, respectively). Administration of both Votucalis and mepyramine had no visible effect on the total time the animals spent exploring the objects (**Figure 8C**; $p > 0.05$).

In addition, systematic (i.p.) administration of Votucalis (20 mg kg^{-1}), either single or chronic, did not impair short-

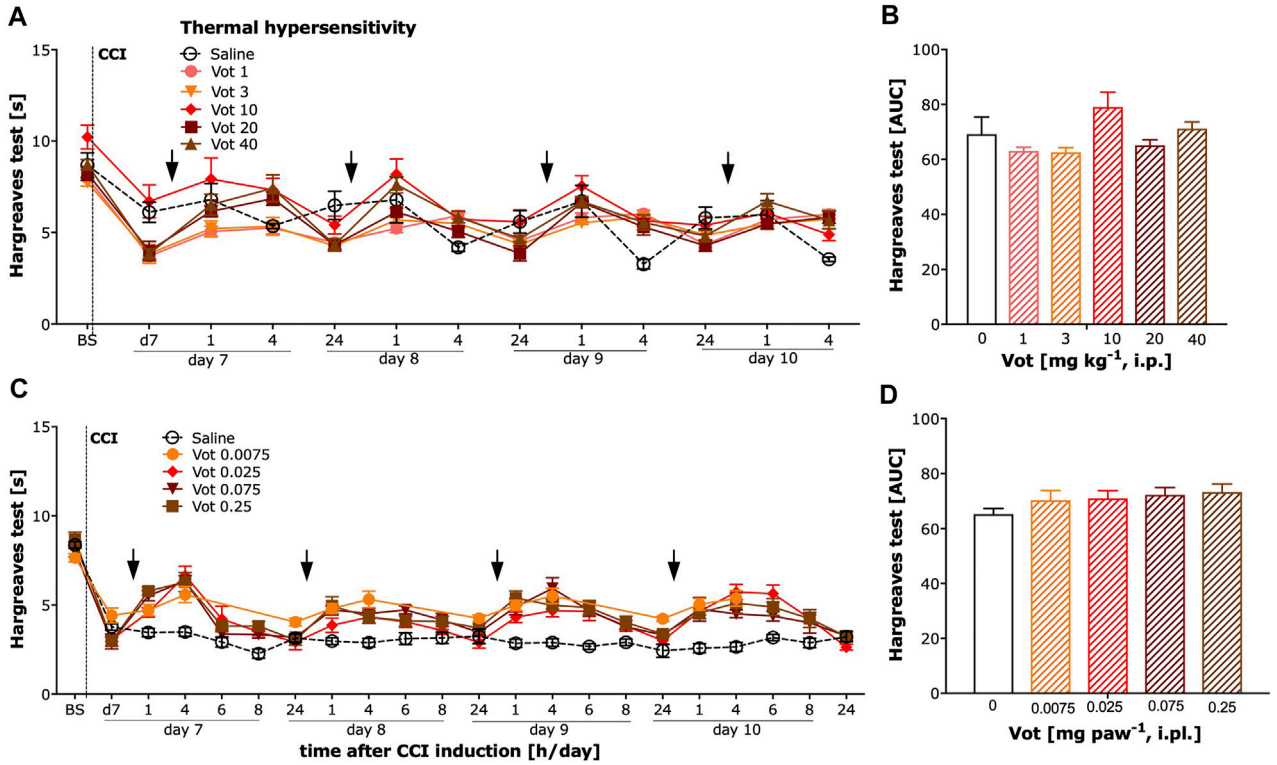

FIGURE 5 | Chronic systemic administration of Votucalis produced a weak effect while peripheral administration of Votucalis did not attenuate thermal (heat) hypersensitivity in the chronic constriction injury (CCI) model of neuropathic pain in mice. Left panel **(A,C)** Time-course effect of systemic (i.p.) injection of Votucalis [Vot, 1–40 mg kg^{-1}, $n = 6$; **(A)]** and peripheral (i.pl.) injection of Votucalis [Vot, 0.0075–0.25 mg paw^{-1}, $n = 6$; **(C)]** administered into the plantar surface of the injured paw, or vehicle [saline, $n = 6$; **(A,C)]**, on the thermal (heat) withdrawal threshold measured with the Hargreaves test. The measurements were assessed before injury as basal pain threshold (BS), and then 7 days following the injury (d7). The effect of Votucalis was assessed 0.5–24 h after each of four consecutive once daily (arrow) intraperitoneal **(A)** or intraplantar **(C)** injections. Data are presented as means ± SEM. *Right panel* **(B,D) (B)** The area under the curve (AUC) summarizing measurements in A. **(D)** The area under the curve (AUC) summarizing measurements in C. One-way ANOVA in B $^*p > 0.05$ but Bonferroni's comparison post-hoc test non-significant vs. vehicle control animals. In D lack of significance vs. vehicle control animals.

term recognition memory and had no effect on the balance and coordination of naïve mice as measured using the rotarod test ($p > 0.05$; data not shown).

DISCUSSION

In this study, we demonstrated that Votucalis, a novel CNS-sparing and high-affinity recombinant histamine binding protein, produced both anti-pruritic and anti-nociceptive effects in mouse models of acute itch and chronic neuropathic pain following both systemic and peripheral administration, which represent commonly used routes of drug applications in clinical practice. The main finding, however, highlights the advantage of peripheral transdermal administration of Votucalis, as the anti-pruritic and anti-nociceptive effects were achieved at significantly lower doses and produced longer duration of effect than systemic administration. Peripheral route of administration was also free from CNS-mediated (side) effects. Thus, this observation provides the first evidence for therapeutic targeting peripherally released histamine by Votucalis to deliver a novel strategy (histamine capture) for potentially safer and more efficacious control of conditions that are known to be regulated by histamine.

Votucalis Attenuated Histaminergic Itch

Our study showed that both systemic and peripheral transdermal administrations of Votucalis attenuated scratching behaviour associated with activation of a histamine-dependent mechanism, as compound 48/80 is a well-known inducer of histamine-dependent itch (Inagaki et al., 2002; Obara et al., 2015). Intracutaneous injection of this compound is known to cause mast cells degranulation resulting in histamine release from human, dog and rodent cutaneous mast cell in a concentration-dependent fashion *via* a mechanism involving activation of histamine receptors. Activation of all these receptors expressed in primary sensory neurons results in the activation of phospholipase A2 (PLA2), phospholipase C-β3 (PLCβ3) and protein kinase C δ (PCKδ) leading to increase of intracellular Ca^{2+} in primary sensory neurons and DRG neurons *via* transient receptor potential subfamily (TRPA1/V1) (Barrett et al., 1985; Tomoe et al., 1992; Bell et al., 2004; Kim et al., 2004; Shim et al., 2007; Liu et al., 2013; Wilzopolski et al., 2021). Histamine released

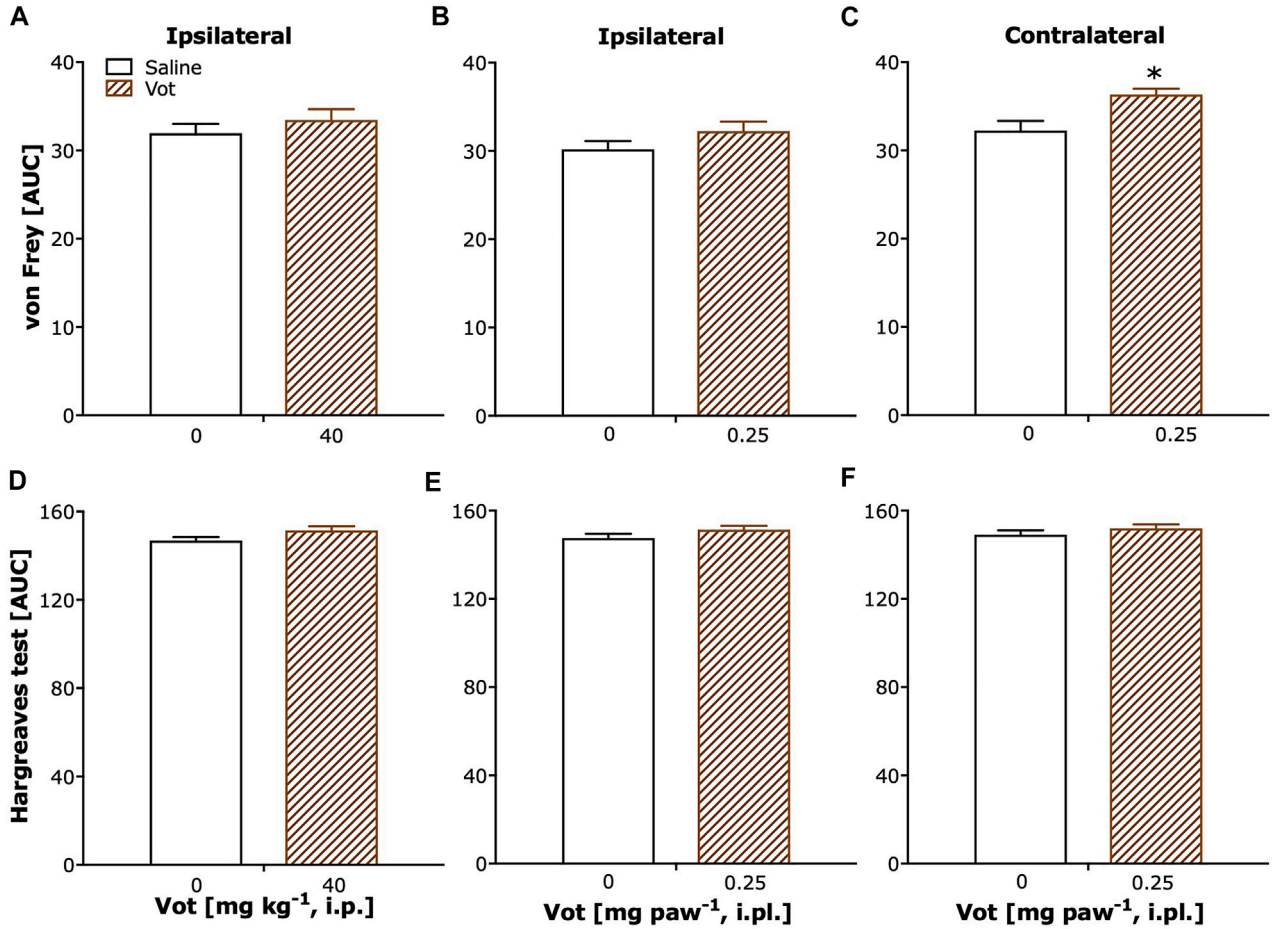

FIGURE 6 | Chronic systemic and peripheral administration of Votucalis did not attenuate overall mechanical or thermal sensitivity in sham mice. **(A–C)** The area under the curve (AUC) summarizing time-course effect of systemic (i.p.) injection of Votucalis [Vot, 40 mg kg^{-1}, $n = 8$; **(A)]** and peripheral (i.pl.) injection of Votucalis [Vot, 0.25 mg paw^{-1}, $n = 8$; **(B,C)**], or vehicle [saline, $n = 5$; **A–C)**], on the mechanical withdrawal threshold measured with von Frey filaments. **(D–F)** The area under the curve (AUC) summarizing time-course effect of systemic (i.p.) injection of Votucalis [Vot, 40 mg kg^{-1}, $n = 8$; **(D)**] and peripheral (i.pl.) injection of Votucalis [Vot, 0.25 mg paw^{-1}, $n = 8$; **(E,F)**], or vehicle [saline, $n = 5$; **(D–F)**, on the thermal withdrawal threshold measured with the Hargreaves test. The measurements were taken on the ipsilateral paw **(A,B,D,E)** or contralateral paw **(C, F)** similarly as described in **Figures 4**, **5** (detailed data not shown). The asterisk (*) denotes significance *vs.* vehicle control animals; $^*p < 0.05$ (two-tailed unpaired *t*-test).

from activated mast cells locally within the dermis, causes leakage of plasma into surrounding tissues and cellular infiltration of neutrophils and eosinophils (Tiligada and Ennis, 2020). Also, in experimental conditions intradermal injection of histamine was shown to produce itch in humans and rodents (Heyer et al., 1997; Laidlaw et al., 2002). Since the crystal structure of Votucalis revealed a high-affinity site for histamine binding (Paesen et al., 1999; Paesen et al., 2000), it is very likely that Votucalis neutralised endogenously released histamine, resulting in a potent and dose-dependent anti-itch effects. The anti-itch effect of Votucalis was particularly profound after peripheral transdermal administration. Specifically, subcutaneous delivery of Votucalis directly to the area affected by itch was effective over the whole 40-min observation period, at a dose over 60-fold lower compared to the lowest systemic dose, that was effective; notably this was only effective during the first 20 min from induction of itch. The striking difference in the potency and efficacy of Votucalis observed when the drug was administered transdermally to the area of itch sensation vs. systemically, further emphasises the nature of Votucalis, as a non-brain-penetrating, highly potent histamine local scavenger. It seems likely that the ability of Votucalis to sequester histamine, subsequently suppressed the binding of histamine to all histamine receptors potentially leading to decrease in firing and excitability of the itch-specific primary afferents, in particular Aδ- and C-fibres, that resulted in attenuation of histaminergic itch (LaMotte et al., 2014; Obara et al., 2015). To further confirm that the anti-itch effect of Votucalis was mediated by neutralisation of histamine, we identified that Votucalis did not reduce scratching behaviour caused by chloroquine that is a Mas-related G protein-coupled receptor A3 (MrgprA3) agonist that upon intracutaneous administration elicits scratching behavior resulting from activation of MrgprA3+ primary sensory neurons that were shown to be essential for itch

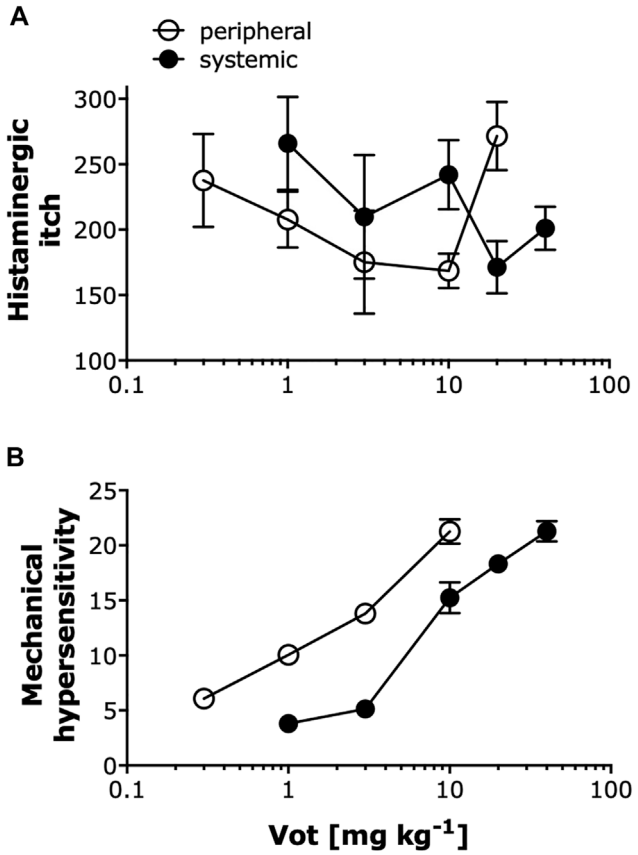

FIGURE 7 | Peripheral administration of Votucalis produced anti-pruritic and anti-nociceptive effects at a lower dose range compared to systemic administration. **(A)** Dose-response effect of Votucalis (Vot, 0.3–40 mg kg^{-1}; i.p. or s.c.) in histamine-dependent itch induced by injection of compound 48/80. Points represent the total number of scratches across a 40-min observation period for each treatment based on the data presented in **Figures 1**, **2**. Data are presented as means ± SEM, n = 6–18 in each group. **(B)** Dose-response effect of Votucalis (Vot, 0.3–40 mg kg^{-1}; i.p. or i.pl.) in the chronic constriction injury (CCI) model of neuropathic pain. Points represent the area under the curve (AUC) presented in **Figure 4** and referring to changes in the mechanical withdrawal threshold measured with von Frey filaments. Data are presented as means ± SEM, n = 6–14 in each group.

sensation (Shiratori-Hayashi et al., 2019). Thus, we showed Votucalis ineffectiveness for histamine-independent itch. However, it should be noted that the highest dose of Votucalis used to inhibit itch was less effective compared to the efficacy of lower doses of Votucalis, potentially revealing a biphasic or bell-shape dose response curve produced by Votucalis. Given that histamine plays very diverse physiological roles, this observation may suggest that higher doses of Votucalis may activate mechanisms that conversely promote itch sensation (possibly *via* the H_3 receptor) and, therefore, counteract the scavenging ability of Votucalis. This may require further investigation. Note, that the anti-itch effects of Votucalis was incomplete, consistent with the high non-physiological levels of histamine released by compound 48/80 in the itch study.

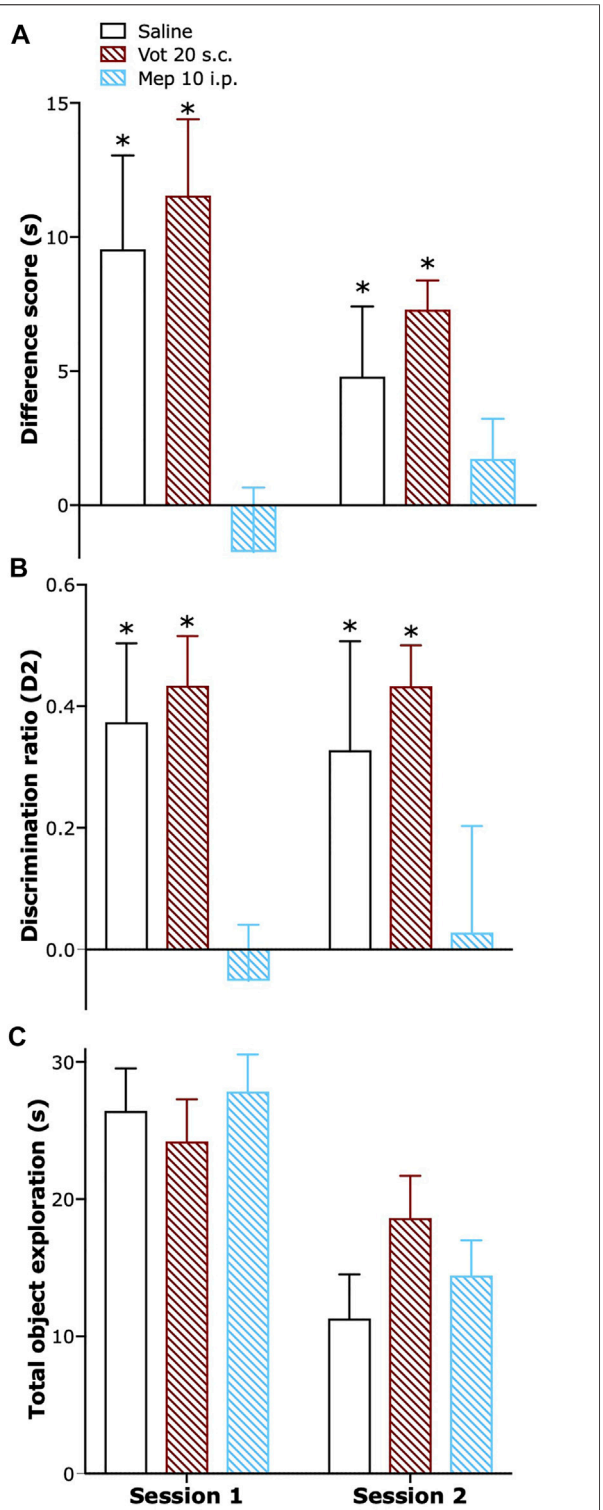

FIGURE 8 | Peripheral administration of Votucalis did not affect short-term recognition memory in naïve mice. **(A)** Time-course effect of a single (session 1) and chronic (session 2) peripheral (s.c.) injection of Votucalis (Vot), or systemic (i.p.) injection of H_1 receptor (H_1R) antagonist mepyramine (Mep) or vehicle (saline), on the performance in the spontaneous novel object recognition task. Mice received a single or chronic (across four consecutive

(*Continued*)

FIGURE 8 | days) injection of Votucalis (20 mg kg^{-1}; $n = 8$), mepyramine (10 mg kg^{-1}; $n = 8$) or vehicle (saline; $n = 8$) 30 min before the task; behavior was recorded and scored offline. Performance in the task is shown as a difference score (mean time spent exploring the novel objects minus the mean time spent exploring the familiar objects) in session 1 and 2. Scores above zero indicate a novelty preference. **(B)** Performance in the task is also shown as a discrimination ratio (difference score divided by mean total time spent exploring the objects in the test phase) for session 1 and 2. **(C)** Total duration of object exploration in the test phase for session 1 and 2. Data are presented as means ± SEM, $n = 8$ in each group. The asterisk (*) denotes significance vs. mepyramine treated animals; $^*p < 0.05$ (one-way ANOVA, followed by Bonferroni's comparison post-hoc test).

Peripheral H_1 Receptor, Peripheral H_2 Receptor and Central H_4 Receptor Mediated Histaminergic Itch as Well as Votucalis Anti-Itch Effects

H_1 receptor antagonists are widely used to relieve itch, however their therapeutic efficacy is limited (Shim and Oh, 2008), indicating that different histamine receptors may be involved in the mediation of itch (Kollmeier et al., 2014). Herein, by using selective histamine receptors antagonists and different routes of their administration we were able to distinguish, for the first time, the potential involvement of peripheral and central histamine receptors in itch. Our study indicates that histaminergic itch is predominantly mediated by peripheral H_1 and H_2 receptors, and less by central H_1 and H_2 receptors, as peripherally administered selective antagonists of these receptors inhibited histaminergic itch at doses that were not effective with systemic administration. Thus, this may suggest the involvement of predominantly peripheral H_1 and H_2 receptors however further investigations may be required to confirm this effect. Nevertheless, these findings provide new insight into the mechanism of the H_1 receptor mediated anti-itch effect as some studies have argued that the anti-itch effect resulting from H_1 antagonism is due to sedation, rather than the direct blockade of H_1 receptor on sensory neurons (Imaizumi et al., 2003; Bell et al., 2004). In line with our observation, both mRNA and functional expression of H_1 receptor were shown on peripheral neurons displaying characteristics of C-fibers (Kashiba and Senba, 2001; Rossbach et al., 2011). In addition, Bell et al. (2004) found that intradermal administration of a H_1 agonist caused dose-dependent scratching in mice.

Our findings also extend the understanding of the role of H_2 receptors in itch, as the available literature is rather inconclusive (Bell et al., 2004; Rossbach et al., 2011). It seems that H_2 receptor-mediated anti-itch response is due to antagonism of predominantly peripherally expressed H_2 receptors that are known to be present on primary afferent neurons (Kajihara et al., 2010), although their functional expression has not yet been confirmed (Rossbach et al., 2011). The proposed role of peripheral H_1 and H_2 receptors in itch transmission was further emphasised by the use of Votucalis as the anti-itch effects was stronger when antagonists targeting peripheral H_1 and H_2 receptors were co-administered with the drug.

In line with other *in vivo* studies, including experiments using knockout animals, we found that antagonism at H_4 receptor suppressed histaminergic itch (Dunford et al., 2007; Rossbach et al., 2011; Wilzopolski et al., 2021). Interestingly, this effect was observed after both peripheral and systemic administration of a H_4 receptor antagonist, however only systemically administered H_4 antagonist produced stronger anti-itch effect when co-administered with Votucalis. We, therefore, suggest that central H_4 receptors may be predominantly involved in the regulation of histaminergic itch.

Votucalis Attenuated Mechanical Neuropathic Pain

Our study showed that both systemic and peripheral transdermal administrations of Votucalis almost completely blocked mechanical hypersensitivity in neuropathic mice. Similarly, as in the itch study, the anti-nociceptive effect of Votucalis was most potent after peripheral transdermal administration; the lowest anti-nociceptive peripheral effective dose was 10-fold lower in comparison to the lowest effective systemic dose. This Votucalis-induced effect highlights the importance of histamine for the maintenance of neuropathic pain symptoms in the periphery where histamine is known to produce nociceptive effects (Yue et al., 2014; Khalilzadeh et al., 2018). Indeed, as a result of tissue injury or damage, histamine released from neuronal and non-neuronal cells, in close proximity to sensory fibers, contributes to the development and maintenance of mechanical and thermal hypersensitivity *via* sensitizing peripheral polymodal nociceptors, which results in increased firing rates and generate action potentials in the neurons (Khalilzadeh et al., 2018; Obara et al., 2020). In addition, it was shown that histamine contributes to neuropathic pain mechanism by increasing voltage-gated Na^+ channels, in particular Nav1.8 and Nav1.9 expression in primary afferent neurons and L4/L5 DRG neurons (Yue et al., 2014; Bennett et al., 2019). Thus, it seems that Votucalis efficiently neutralized endogenously released histamine causing a potent and dose-dependent anti-nociceptive effect. Interestingly, however, while mechanical hypersensitivity was blocked by peripherally administered Votucalis, heat hypersensitivity remained unaffected by this treatment. This modality-specific anti-nociceptive effect may suggest involvement of H_3 receptors and that Votucalis prevents histamine binding to H_3 receptor since both pharmacological and genetic manipulations of H_3 receptor activity have confirmed its importance and specificity for mechanical hypersensitivity (Cannon et al., 2003; Wei et al., 2016). In addition, anatomical studies have confirmed localization of H_3 receptors on Aδ-fibers that conduct tactile sensation (Lawson, 2002; Cannon et al., 2007) as well as the ability of H_3 antagonists to block secondary mechanical hypersensitivity (Medhurst et al., 2007). It may suggest that Votucalis, by scavenging endogenous histamine, reduced the sensitivity of H_3-positive A-fibers resulting in a diminished input to the dorsal horn, supporting the potential role for H_3

receptors in the modulation of central sensitization. In contrast, heat hypersensitivity is regarded as a sign of the peripheral sensitization of C-fibers, which do not express H_3 receptors (Cannon et al., 2007). This C-fibre sensitization may be due to the involvement of H_4 receptors (Obara et al., 2020).

In summary, this is the first report showing that targeting histamine itself, by sequestering the endogenous ligand within Votucalis, may represent a new tool to control conditions that are known to be regulated by peripherally released histamine. It should be, however, noted that future studies should attempt to quantify the efficacy of histamine scavenging by Votucalis. Nevertheless, *in vivo* neutralization of locally administered Votucalis has also shown previously a wide range of anti-inflammatory effects in mouse models of acute respiratory distress syndrome (ARDS) or allergic asthma (Ryffel et al., 2005). Herein, our new approach may provide many therapeutic advantages over drugs targeting histamine receptors, which when tested for their utility in attenuating itch and pain, have shown inconsistent and limited efficacy. Our studies, using the spontaneous novel object recognition task, suggest that local peripheral sequestration of histamine by Votucalis may have therapeutic potential as a non-sedating and non-addictive analgesic agent. The use of analgesics that act at the peripheral level is justified in accordance with current concepts in pain medicine, which emphasize the importance of an individualized and mechanism-based approach in pain management (Müller-Schwefe et al., 2017; Kocot- Kępska et al., 2021). It is expected that peripherally (e.g., transdermally) applied analgesics will only target the underlying molecular/cellular mechanisms in the periphery, negating the need to consider systemic mechanisms and, therefore, their long-term use may be safer, as well as effective.

DATA AVAILABILITY STATEMENT

The raw data supporting conclusion of this article is available at https://doi.org/10.25405/data.ncl.19203461.

ETHICS STATEMENT

Experimental protocols (PPL: P8E3496FA, P6694C943) were performed under UK Home Office license, with AWERB local approval, and in accordance with current UK legislation as defined in the Animals (Scientific Procedures) Act 1986. The ARRIVE guideline has been followed in reporting this study.

AUTHOR CONTRIBUTIONS

IA conducted the studies, analyzed the data and wrote the main draft of the manuscript; EB and AA conducted parts of the studies; EB also reviewed the manuscript; MC conducted and analyzed parts of the studies, and wrote parts of the manuscript; AE designed and analyzed parts of the studies; WW-D and MN reviewed the final draft of the manuscript; PLC designed the experiments and reviewed the manuscript; IO designed the experiments, analyzed the data and wrote the manuscript.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.