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João Guerreiro Covita

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THÈSE EN COTUTELLE PRÉSENTÉE
POUR OBTENIR LE GRADE DE

DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ET DE L'UNIVERSITÉ DE MELBOURNE

ÉCOLE DOCTORALE UBX
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SPÉCIALITÉ NEUROSCIENCE

Par João COVITA

**ROLE OF RELAXIN-3/RXFP3 FOREBRAIN NETWORKS IN
DESCENDING CONTROL OF PAIN IN THE MOUSE**

Sous la direction de Marc LANDRY
et de Andrew GUNDLACH

Soutenue le 15 Juillet 2019

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Titre:

Résumé : La douleur est un processus biologique complexe qui alerte sur les changements potentiellement délétères de l'environnement et s'avère nécessaire à la survie de l'organisme. Toutefois, l'activation continue de ce système de signalisation peut provoquer des changements maladaptifs qui se caractérisent par des altérations anatomiques et fonctionnelles. La douleur est considérée comme chronique lorsque sa durée dépasse 3 mois. Les douleurs chroniques sont accompagnées de problèmes économiques et sociaux qui font de leur traitement une priorité de santé publique.

Le cerveau décode les composantes sensori-discriminatives et affectives de la douleur. Il détermine ainsi le type de douleur (pincement, brûlure, etc.), et sa localisation dans l'organisme, et sa valeur aversive, respectivement. Le cerveau transmet ensuite à la périphérie des informations en accord avec les stimuli reçus, et qui tiennent aussi compte des expériences présentes et passées de l'individu.

Le signal nociceptif est modulé à toutes les étapes de sa transmission par la mise en jeu de nombreux systèmes neurochimiques, et en particulier par des neuropeptides. La présence de neuropeptides et de leurs récepteurs dans des aires du système nerveux central (SNC) impliquées dans la transmission douloureuse suggère que les systèmes peptidergiques participent au contrôle de la nociception.

La relaxine-3 est un neuropeptide principalement synthétisée dans une région du rhombencéphale appelée Nucleus Incertus (NI). Depuis sa découverte, la relaxine-3 a été impliquée, via l'activation de son récepteur RXFP3, dans le contrôle d'une large variété de comportements tels que l'anxiété, l'éveil, la recherche de récompense. Ces rôles de la signalisation relaxine-3/RXFP3 suggèrent que l'activation du récepteur RXFP3 pourrait contribuer à la modulation de la sensibilité douloureuse.

Mes travaux visaient initialement à évaluer les effets de l'activation et de l'inhibition du récepteur RXFP3 sur la sensibilité thermique et mécanique dans des conditions normales et en situation de douleurs persistantes. Nos travaux ont démontré que l'administration centrale, par injection intracérébroventriculaire (icv), de l'agoniste peptidique A2 du récepteur RXFP3, provoquaient une diminution de la sensibilité mécanique mais pas thermique. De plus, l'injection icv de l'antagoniste peptidique R3(B1-22)R augmente la sensibilité mécanique et thermique. Ces données suggèrent que la signalisation relaxine-3/RXFP3 exerce un effet tonique inhibiteur sur la sensibilisation mécanique et thermique, et que la mise en jeu du récepteur RXFP3 pourrait avoir des propriétés antalgiques.

De plus, j'ai étudié la mise en jeu de différentes aires cérébrales en comparant l'immunoréactivité c-Fos dans différentes régions après injection de l'agoniste A2. Toutefois, ces travaux n'ont pas révélé de différence dans le nombre des cellules marquées ni dans l'intensité du marquage.

L'hybridation in situ de différentes aires cérébrales a permis d'identifier les populations exprimant les ARNm de RXFP3. J'ai analysé la coexpression des ARNm de marqueurs spécifiques, parvalbumine et somatostatine, et déterminé l'expression relative des ARNm de RXFP3 parmi ces populations.

Enfin, j'ai examiné une possible anxiété comorbide chez des souris en conditions de douleur inflammatoire. De manière surprenante, le comportement anxieux n'était pas modifié chez ces souris suggérant que ce modèle ne développe pas d'anxiété et que l'activation du RXFP3 cible spécifiquement la transmission nociceptive.

L'ensemble de mes résultats montre l'implication du système relaxine-3/RXFP3 dans le contrôle de la douleur, offrant de nouvelles possibilités pour le développement d'outils thérapeutiques en ciblant un système peptidergique susceptible d'impacter plusieurs comportements altérés en conditions de douleurs chroniques.

Mots clés : Relaxine-3, RXFP3, douleur, contrôles descendants

Title: Role of relaxin-3/RXFP3 forebrain networks in the descending control of pain in the mouse

Abstract: Pain is a complex biological phenomenon that is beneficial and necessary for our survival, warning of changes and hazards in the environment that compromise optimal function. However, continuous activation of pain signalling systems results in maladaptive changes characterized by altered tissue and organ structure and activity. Pain lasting more than 3 months is termed chronic pain and it is under these conditions that it becomes a major burden for affected individuals. Chronic pain is also accompanied by serious social and economic burdens, making research in this field a high priority globally.

The central nervous system (CNS) acts as a major control centre for nociceptive signal transmission, decoding pain for its sensory-discriminative and aversive components, i.e., deciphering the type of pain (pinch, burn, etc.), its location in the body, and its associated hedonic value, respectively. Once the information is decrypted, neural signals from the brain to the periphery act in accordance with the provided stimulus based on past and current experiences.

The nociceptive signal is modulated at every step of this process by an abundance of neurochemical signals, including neuropeptides. The presence of neuropeptides and/or their receptors in areas linked to nociceptive processing and transmission suggests putative roles for these systems in the control of nociception.

Relaxin-3 is a neuropeptide that is mainly synthesized in a hindbrain region known as the nucleus incertus (NI). Since its discovery, relaxin-3 has been linked to the control of a wide range of behaviours such as anxiety-like behaviours, arousal, and reward-seeking, through activation of the Gi/o-protein-coupled receptor, RXFP3. These putative roles of relaxin-3/RXFP3 signalling suggest a possible link between RXFP3 activation and modulation of pain sensitivity.

Therefore, my initial studies assessed the effect of RXFP3 activation and inhibition on mechanical and thermal pain sensitivity in normal and persistent pain conditions. These studies demonstrated that central administration of the RXFP3 agonist peptide, RXFP3-A2, via intracerebroventricular (icv) injection, produced relief of mechanical, but not thermal, pain sensation. Moreover, icv injection of the RXFP3 antagonist peptide, R3(B1-22)R, augmented mechanical and thermal pain sensitivity. These data suggest that relaxin-3/RXFP3 signalling has a tonic action in maintaining mechanical and thermal pain thresholds, and the potential for activation of RXFP3 to produce pain relief.

Additionally, I examined the possible involvement of different brain areas in these effects, by assessing the number of c-Fos-positive cell nuclei under different conditions. However, these studies revealed no difference in the number of c-Fos-positive cell nuclei or staining intensity in the vehicle- and RXFP3 agonist-treated mice.

Further characterization of pain circuit-related brain areas using multiplex in situ hybridization revealed that RXFP3 mRNA is expressed within discrete populations of neurons in these areas. I also evaluated possible co-expression of RXFP3 mRNA with somatostatin and parvalbumin mRNA, and determined the relative proportion of RXFP3 mRNA expression in populations of neurons that express these transcripts.

Finally, I examined the possible presence of comorbid anxiety in mice subjected to the persistent pain protocol. However, anxiety-like behaviour was not altered in mice with persistent hindpaw pain, suggesting this model does not display produce anxiety, and that effects of RXFP3 modulation observed specifically targeted nociceptive transmission.

Overall, my findings implicate the relaxin-3/RXFP3 system in control of pain transmission, providing new opportunities for the development of therapeutic tools for pain management, by targeting a neuropeptide system that impacts several behaviours that are altered in chronic pain conditions.

Keywords: Relaxin-3, RXFP3, pain, descending controls

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Declaration

This is to certify that:

- this thesis comprises only my original work towards the degree of Doctor of Philosophy, except where indicated.
- due acknowledgment has been made in the text of all material used, and contributions of others.
- the thesis complies with the regulations set for the degree of Doctor of Philosophy, following the agreement between the University of Bordeaux and The University of Melbourne leading to the award of a joint doctorate.

Signed,

João Miguel Guerreiro Covita

October 2019

Preface

I would like to acknowledge the following people for their intellectual input, help with data collection, and technical support during my candidature. Firstly, I would like to thank my principal supervisors, Professor Marc Landry and Professor Andrew Gundlach, who devised this novel project, and provided intellectual guidance throughout my academic journey; and my co-supervisor, Dr Sherie Ma, who assisted me with the pharmacological studies in rats and taught me the ‘art’ of multiplex *in situ* hybridization histochemistry. I would like to thank Professor Gundlach and Dr Rabia Bouali-Benazzouz for preparing the ethics applications related to my Florey and Bordeaux studies, respectively, and Dr Fabrice Cordelières and Dr Sébastien Marais for their guidance on confocal microscopy. I would also like to thank Ms Maude Wagner for performing the statistical analysis of immunohistochemistry data.

I would also like to acknowledge the generous support of a Melbourne International Fee Remission and Melbourne International Research Scholarship and a scholarship from Initiative d’Excellence (IdEx).

I declare that this thesis contains no material which has been accepted for the award of another degree or qualification, and no previously published material or material written by another person or research conducted prior to my PhD candidature enrolment, except where duly referenced.

Publications

Roca-Lapirot O, Fossat P, Ma S, Egron K, Trigilio G, López-González MJ, **Covita J**, Bouali-Benazzouz R, Favereaux A, Gundlach AL and Landry M. (2019) Acquisition of analgesic properties of the cholecystokinin/CCK₂ receptor system within the amygdala in a persistent inflammatory pain condition. *Pain* **160**, 345-357.

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XV Meeting for the Portuguese Society of Neurosciences (May 2017) – **Covita J**, Bouchatta O, Aby F, Fossat P, Benazzouz, Ma S, Gundlach AL, Landry M. Peptidergic modulation of pain and anxiety: Forebrain relaxin-3/RXFP3 networks and descending control of nociception in mice.

Biennial Meeting of the International Society of Neurochemistry (August 2017) – **Covita J**, Bouchatta O, Aby F, Fossat P, Bouali-Benazzouz R, Ma S, Gundlach AL, Landry M. Peptidergic modulation of pain and anxiety: Forebrain relaxin-3/RXFP3 networks and descending control of nociception in mice.

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Travel Award – 1st Prize for 2nd Year PhD students, Florey Student Talks, The Florey Institute for Neuroscience and Mental Health (Melbourne, Australia, June 2017)

Travel Award – International Society for Neurochemistry (Paris, France, August 2018)

Travel Award – 3rd Prize, Students of Brain Research Symposium, The Florey Institute for Neuroscience and Mental Health (Melbourne, Australia, October 2017)

Travel Award – Federation of European Societies for Neuroscience (FENS), awarded by the Portuguese Society for Neuroscience (Berlin, July 2018)

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A doctoral thesis is a compilation of scientific research and focuses on one key aspect of its author's life. It does not, however, fully reflect the journey traveled, the progress made; or account for those people who influenced the work, directly or indirectly. Many aspects of my life were changed over the last three and a half years, and this section of my thesis is dedicated to the people who helped me grow as a person and a scientist.

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Dedication

I dedicate this thesis to all the women in my life dealing with breast cancer

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List of Abbreviations

5-HT	5-hydroxytryptamine
AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
aCSF	Artificial cerebrospinal fluid
ADT	Anterodorsal nucleus of the thalamus
amBNST	Anteromedial part of the bed nucleus of the stria terminalis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Anteroposterior
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
BOLD	Blood-oxygen-level dependent
bp	Base pairs
CB	Calbindin
CCI	Chronic constriction injury
CeA	Central amygdala
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CPN	Common peroneal nerve
CRF	Corticotropin-releasing factor
CVLM	Caudal ventrolateral medulla
DH	Dorsal horn
DRG	Dorsal root ganglion
DV	Dorsoventral
ENK	Enkephalin
EPM	Elevated plus maze
EPSC	Excitatory postsynaptic current
ERK1/2	Extracellular signal-regulated kinase 1/2
GAL	Galanin

GnRH	Gonadotropic-releasing hormone
GPCR	G protein-coupled receptor
HCN	Hyperpolarization-activated cyclic nucleotide-gated
HPLC	High-performance liquid chromatography
i.p.	Intraperitoneal
icv	Intracerebroventricular
INSL5	Insulin-like peptide 5
LA	Lateral amygdala
LDB	Light-dark box
LH	Luteinizing hormone
LPB	Lateral parabrachial nucleus
LV	Lateral ventricle
MALDI-TOF	Matrix-assisted laser desorption/ionization-time-of-flight
mGluR	Metabotropic glutamate receptor
ML	Mediolateral
NaCl	Sodium chloride
NGF	Nerve growth factor
NI	Nucleus incertus
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OT	Oxytocin
PAG	Periaqueductal grey
PB	Parabrachial nucleus
PCR	Polymerase chain reaction
PH	Posterior hypothalamus
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C

PnR	Pontine raphe nucleus
PT	Parataenial nucleus of the thalamus
PV	Parvalbumin
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
PWL	Paw withdrawal latency
PWT	Paw withdrawal threshold
RVM	Rostroventral medulla
RXFP3	Relaxin family peptide receptor 3
RXFP3 KO	RXFP3 knockout
s.c.	Subcutaneous
SNI	Spared nerve injury
SNL	Spinal nerve ligation
SP	Substance P
TrkA	Tropomyosine receptor kinase A
TRPV1	Transient receptor potential cation channel subfamily V member 1
UV	Ultraviolet
VGLUT3	Vesicular glutamate transporter type 3
vIPAG	Ventrolateral part of the periaqueductal grey

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Chapter 1 - General introduction

1.1 Preface

Pain is a biologically complex phenomenon characterized by both the sensation it causes when a tissue is stimulated beyond its normal, functioning thresholds, and the feeling it evokes in the subject. Understanding pain signalling encompasses the study of spinal cord and brain circuits that process nociceptive information at different levels, spanning neuronal transmission to molecular signalling. The published literature on pain is wide in both scope and depth, with studies focusing on different components of the nociceptive system in a variety of pain models (e.g., computational or animal-based models) and on a huge range of specific molecular targets to manipulate pain thresholds and understand associated mechanisms. The complexity of these broad studies and their interpretation increases further when we consider the many behaviours with which pain interacts. Indeed, several types of behaviours are modulated by the activity of brain structures that also influence pain. This translates into maladaptive changes in these circuits in the context of persistent pain, leading to several comorbid states in chronic pain patients. Thus, exploring the mechanisms involved in pain and pain-related behaviour processing, as well as identifying therapeutic tools for chronic pain management, has become a prominent and important field of research in recent years. My research has focused on a neuropeptide system and its ability to modulate pain-related behaviours, as well as its role in descending nociceptive transmission. I also conducted studies that sought to identify the relevant brain structures involved in these processes. Additionally, I evaluated the expression of anxiety-like behaviours in the context of short-lasting and persistent pain.

This Chapter provides a summary of the basic concepts of nociceptive transmission, and the terminology and methodology used in this body of research. I will review the concept of pain, other terms and concepts used throughout this thesis, and the anatomy of structures involved in ascending and descending pain transmission.

1.2 Pain

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bogduk, 1994). It arises in the presence of noxious stimuli of varied natures that can be divided into physical (i.e., heat, cold, pressure, pinch) and chemical (i.e., acid, irritants, inflammatory compounds) stimuli. Some other important concepts are summarized in

Table 1.1.

Table 1.1. Concepts in Pain Research (Merskey and Bogduk, 1994).

Concept	Definition
Allodynia	Pain due to a stimulus that does not normally cause pain
Hyperalgesia	Increased response to a painful stimulus
Analgesia	Absence of pain in response to stimulation which is normally painful
Central pain	Pain initiated or caused by a primary lesion or dysfunction in the central nervous system
Neuropathic pain	Pain initiated or caused by a primary lesion or dysfunction in the peripheral nervous system
Nociceptor	Receptor preferentially sensitive to a noxious stimulus or to a stimulus which becomes noxious if prolonged
Pain threshold	The lowest level of pain a subject can recognize

Pain comprises both a sensory component (the sensation of pain) and an emotional component (the perception of pain). These components can be functionally associated with the ascending and descending controls of pain, respectively.

1.2.1 Ascending pain pathways

Peripheral neurons detect noxious and innocuous stimuli using different types of sensory fibres: A β fibres that are composed of highly myelinated neurons, A δ fibres that are only thinly myelinated and C fibres that are unmyelinated. The level of myelination of these fibres is inversely proportional to their conduction velocities (Harper and Lawson, 1985),

and thus, they have distinct functions. A β fibres convey light touch sensation and therefore are generally non-nociceptive, albeit these fibres can become nociceptive under pain conditions; while A δ and C fibres, convey pain sensation and thus are nociceptive.

The cell bodies of these neurons are located in the dorsal root ganglia (DRG) through which they communicate with different laminae of the dorsal horn (DH) of the spinal cord (**Figure 1.1**). Different nociceptive fibres are sensitive to different mechanical, thermal, and/or chemical stimuli and display distinct neurochemical properties. For example, neurons expressing the transient receptor potential cation channel subfamily V member 1 (TRPV1) convey moderate thermal stimuli (43-55°C), as deletion of the *trpv1* gene abolishes the expression of thermal allodynia in inflammatory pain models without affecting mechanical allodynia (Caterina et al., 2000).

At the level of the spinal cord, complex networks of glutamatergic excitatory neurons and GABAergic/glycinergic inhibitory interneurons work to convey different sensations. Early-life transient vesicular glutamate transporter type 3 (VGLUT3) expression in glutamatergic neurons of the spinal cord is required for the expression of mechanical, but not thermal allodynia (Peirs et al., 2015). Moreover, GABAergic/enkephalinergic (Enk) neurons of the spinal cord set the tone for mechanical sensitivity (François et al., 2017). In neuropathic pain conditions, it has been proposed that mechanical allodynia results from synaptic loss between parvalbumin (PV)-positive interneurons and protein kinase C- γ (PKC γ)-expressing excitatory neurons (Petitjean et al., 2015). These lines of evidence propose that fine tuning of mechanical sensitivity is controlled by diverse mechanisms.

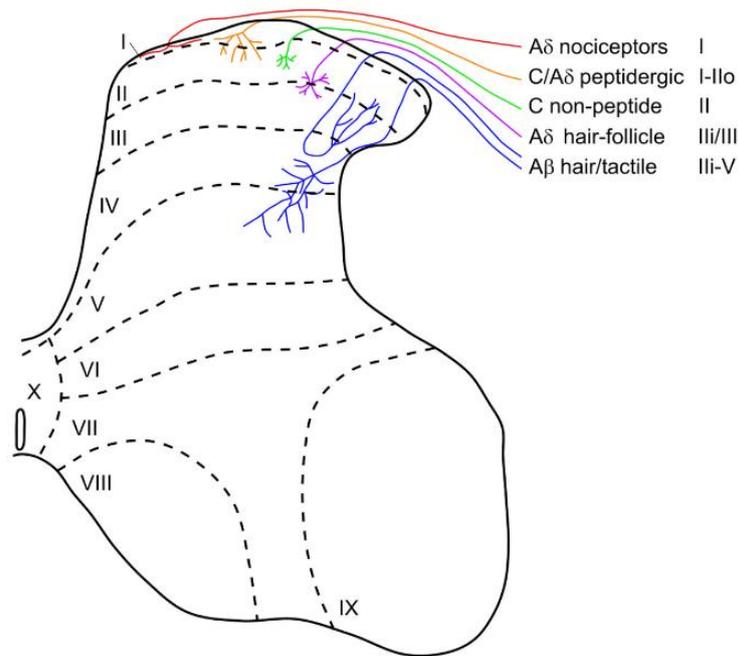


Figure 1.1. Laminar organization of the dorsal horn and primary afferent inputs. The central terminations of the major primary afferent types are shown. A β tactile and hair afferents end mainly in laminae III-VI with some extension into lamina II. A δ hair follicle afferents arborise on either side of the lamina II/III border, whereas A δ nociceptors end mainly in lamina I, with some sending branches to laminae V and X. Myelinated nociceptors with conduction velocities in the A β range arborise throughout laminae I-V (not shown). Peptidergic primary afferents (which also include some A δ nociceptors) arborise mainly in lamina I and II, with some fibres penetrating more deeply, whereas most non-peptidergic C fibres form a band that occupies the central part of lamina II. Figure and legend adapted from (Todd, 2010).

Neurons in the dorsal horn that receive inputs from afferent fibres mainly project to lamina I (LI, **Figure 1.1**), with some projecting to neurokinin 1 receptor (NK1R)-expressing cells in LIII-IV (**Figure 1.2**). These, in turn, project to supraspinal structures involved in pain processing, such as the *nucleus tractus solitarius* (NTS), the caudal ventrolateral medulla (CVLM), the lateral parabrachial nucleus (LPB), the periaqueductal grey (PAG) and the thalamus. Neurons within these areas, in turn, project to different cortical and subcortical regions, which process the affective and sensory-discriminative components of pain and initiate a response to the noxious stimulus.

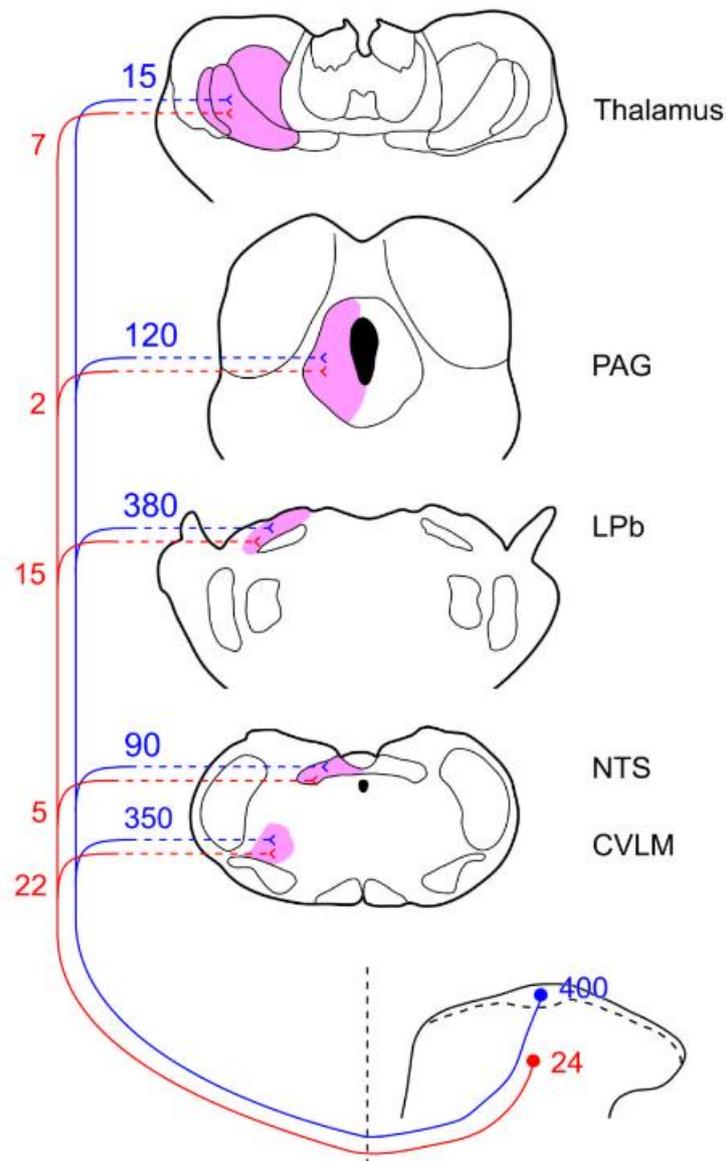


Figure 1.2. Dorsal horn projection neurons. There are approximately 400 projection neurons in lamina I in the rat (~5% of the total neuronal population in this lamina) (blue numbers). Most of these can be retrogradely-labelled following injection of tracer into the lateral parabrachial nucleus (LPB) or caudal ventrolateral medulla (CVLM), with smaller numbers projecting to the other sites. Studies involving paired injections indicate that virtually all lamina I neurons that are retrogradely-labelled following tracer injection into thalamus, periaqueductal grey matter (PAG) or *nucleus tractus solitarius* (NTS) also project to LPB. The numbers of neurokinin 1 receptor (NK1R)-expressing projection neurons in laminae III-IV that can be labelled from each site are also shown (red numbers). There are ~24 of these neurons per side in the L4 segment (corresponding to ~0.1% of the neurons in this lamina). Figure and legend adapted from ref (Todd, 2010).

1.2.3 Descending control of pain

Supraspinal structures receiving inputs directly or indirectly from the spinal dorsal horn are responsible for the processing of pain. Pain processing refers both to the sensory-discriminative and affective-aversive components. In this regard, the sensory-discriminative component is processed by primary and secondary sensory cortices (S1 and S2, respectively) (Gingold et al., 1991), whereas the affective-aversive component is processed by a number of supraspinal structures including, but not limited to, the medial prefrontal cortex (mPFC), the anterior cingulate cortex (ACC), the amygdala and the hypothalamus (**Figure 1.3**). In humans, high pain unpleasantness was linked to increased activity of the ACC, but not S1 or S2 (Rainville et al., 1997) and ACC stimulation has been shown to facilitate nociceptive transmission in rodent models (Calejasan et al., 2000). In the mPFC, a structure associated with affective behaviour, blood-oxygen-level-dependent (BOLD) signal intensity was correlated to spontaneous pain intensity, but this correlation was not present when subjects were submitted to thermal stimulation (Baliki et al., 2006), suggesting this brain region is related to the aversive component of pain, rather than discriminative processing.

Descending pain control is bi-modal, whereby pain is either potentiated (descending facilitation) or suppressed (descending inhibition) and several studies have identified structural changes occurring in different brain regions in pain states, particularly morphological and synaptic plasticity (see Section 5.1.1) and associated changes in affective behaviours (see Section 5.1.2). Higher descending pathways project to the PAG or the rostroventral medulla (RVM) to modulate pain. The PAG projects mainly to the RVM, which projects to the dorsal horn of the spinal cord (Fields and Heinricher, 1985).

The RVM is defined as the midline pontomedullary area that includes the nucleus raphe magnus and the adjacent reticular formation (Heinricher et al., 2009).

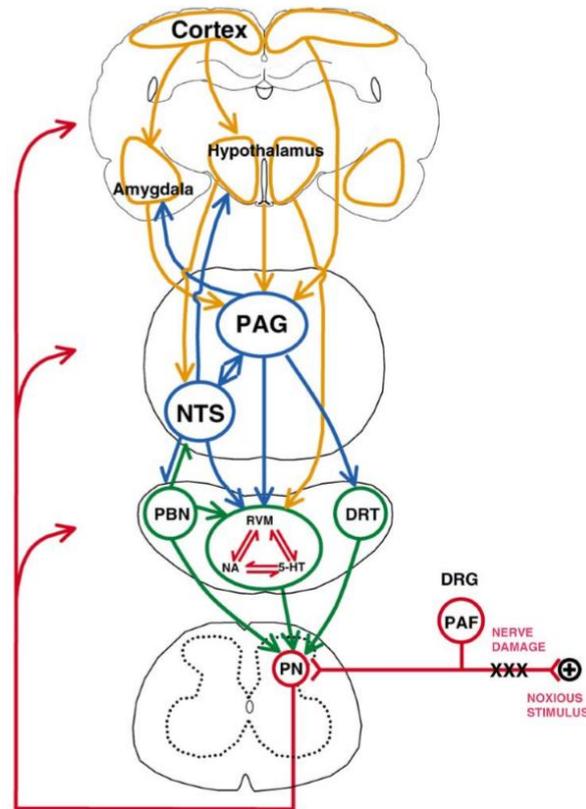


Figure 1.3. Interrelationship between cerebral structures involved in descending modulation of pain.

Direct projections from the cortex, hypothalamus, and nucleus tractus solitarius to the DH are not indicated for clarity - direct pathways to the DH from the periaqueductal grey and amygdala are very sparse. Abbreviations: DRG, dorsal root ganglion; DRT, dorso-reticular nucleus; NA, noradrenaline; NTS, nucleus tractus solitarius; PAF, primary afferent fibre; PBN, parabrachial nucleus; RVM, rostroventral medulla; and 5-HT, serotonin (serotonergic perikarya). Figure and legend adapted from (Millan, 2002).

Chapter 2 – Peptidergic modulation of pain

2.1 Introduction

While my studies focused on a specific neuropeptide (relaxin-3) system, the idea of targeting similar systems is not new to the field of pain research. Indeed, several neuropeptide systems have been explored by other investigators, both past and present. Understanding how each of these systems works to induce or alleviate pain is essential for discerning the translational potential of my own findings.

Neuropeptide systems are diverse in both signalling molecules (neuropeptides) and signalling effectors (receptors), as are the available pain models, and pharmacological tools used for studying the mechanisms through which neuropeptide systems influence pain thresholds; meaning the related literature is equally diverse and wide in scope. For example, most neuropeptide systems signal through G-protein-coupled receptors (GPCRs). As my studies focused on a neuropeptide system – relaxin-3 and its cognate receptor, RXFP3 – that signals through a $G_{i/o}$ -protein coupled receptor, this chapter will review systems that share similar signalling pathways.

Within the pain literature, one family of neuropeptide, the opioids, is most prominent. The following section will review how opioids became the leading analgesic compounds, and the properties that make them poor therapeutic options in a range of pain syndromes. Next, I review evidence that other neuropeptide systems are also viable targets for pain management (Section 2.2).

2.2 Review Article 1

Opioid and non-opioid neuropeptide systems share many signalling effectors through the activation of various GPCRs. Opioid receptor (OR) activation produces rapid pain relief, and opioid drugs are potent therapeutic options for management of short-lasting pain. However, the increasing number of chronic pain patients has led to an increase in prescription of opioid drugs, translating into the misuse of opioid drugs in long-term pain management, and the “opioid epidemic” (Volkow and McLellan, 2016). As such, recent research has shifted its focus to non-opioid neuropeptide systems, to take advantage of the signalling pathways shared with opioid systems. Ideally, next generation painkillers will comprise drugs that can dampen pain intensity as much as pain chronicity, thus limiting the expression of pain comorbidities. It is now apparent that evaluating progress made in this field is of critical importance, to understand the advances that have been made in recent years, as well as identifying the gaps that limit the development and translational potential of emerging therapeutic tools. Thus, with the help of other authors including my PhD supervisors, I prepared a review article, in which these issues are discussed. The main emphasis of the article is the role that different non-opioid neuropeptide systems play in modulating nociception, as well as pain-related behaviours. Additionally, evidence for ‘cross-talk’ between the various non-opioid neuropeptide systems and the opioid systems is summarized, along with progress in the development of pharmacological therapeutic tools for pain management. The latest draft of this review article is provided below.

Title: Targeting non-opioid neuropeptide systems for the management of pain

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1. Introduction

Pain is a complex biological phenomenon that is necessary for survival of animals. It serves beneficial purposes, warning the organism about changes in environment that could compromise proper functioning, and is thus a highly controlled process. However, continuous activation of this signaling system results in maladaptive changes characterized by altered activity and architecture. Pain lasting more than 3 months is termed chronic pain and it is in these conditions that pain becomes a significant burden for the lives of individuals. It is estimated that 19% of the European population suffers from moderate-to-severe chronic pain (Reid et al., 2011), whereas approximately 15% of the United States population suffers from some form of chronic pain (Hardt et al., 2008). Chronic pain is also accompanied by serious social and economic burdens (Smith and Torrance, 2012), making research in this field both prominent and a prevailing necessity.

When considering nociceptive signal transmission, the peripheral nervous system (PNS) can be viewed as both the messenger and effector, as it is responsible for receiving the nociceptive message, which it communicates to the spinal cord via low-myelin A δ and C fibers, and executing actions necessary for survival, such as avoidance. In turn, the spinal cord acts as a second order messenger, by transmitting information to the brain, and signal modulator, as it processes the received information and discerns how and what to transmit to the brain. The brain then acts as a control center, decoding pain for its sensory-discriminative and aversive components, i.e., deciphering the type of pain (pinch, burn, etc.) and its location in the body, and its associated hedonic value (pleasure vs displeasure), respectively. Once the information is decrypted, the brain, via the spinal cord, indicates a way for the PNS to act in accordance to the provided stimulus, based on current and past experiences, and records any new information it has received.

The nociceptive signal is modulated at every step of this process by an abundance of neurochemical players, one of which is neuropeptides. Neuropeptides are small molecules consisting of chains of amino acids that are expressed in neurons and signal to other neurons. They are usually expressed in *prepro* forms, meaning they will undergo some type of processing (e.g. cleavage) before reaching their mature form.

The majority of neuropeptides were first described in the second half of the 20th century. As biochemical and cellular assays were developed over the years, more neuropeptides were discovered using more modernized techniques, allowing the field to progress faster. Most neuropeptides signal with high specificity through G protein-coupled receptors (GPCRs) linked to different types of G proteins. The presence of these peptides and/or their receptors in the central nervous system (CNS) areas linked to nociceptive processing and transmission suggests putative roles for these neuropeptide systems in the control of nociception.

One family of neuropeptides is widely known by researchers, as well as the general public, to affect pain transmission: opioids. This class of neuropeptides has been investigated in the past for its potent antinociceptive properties. However, opioid drugs are not viable options for long-term use, given their addictive properties, the development of tolerance, gastrointestinal discomfort, and other side-effects (Schuckit, 2016). Thus, signaling molecules that use similar mechanisms to produce analgesia, such as non-opioid neuropeptides, present attractive targets for basic research when investigating nociceptive signal modulation and seeking new therapeutic options for pain management.

In this review, we summarize recent advances in this field, and highlight some key features of preclinical studies that contribute to or hinder progress in basic research, in relation to non-opioid neuropeptides that play a role in nociception, with focus given to the development of pharmacological tools for the management of chronic pain.

2. Effects on nociception

The mechanisms through which different neuropeptide systems manipulate nociception, be it the sensory-discriminative or aversive components of pain, remain elusive, are the focus of a large body of research, and have been extensively reviewed individually (Table 1). In this regard, one of the goals of this review is to analyse recent advances that have contributed to the progress of the field.

When considering the role of neuropeptides on nociceptive transmission, most studies focus either spinal or supraspinal modulation of pain. At the spinal level, the pronociceptive neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are commonly used as predictive measures of pain outcomes, whether measured directly through immunohistochemistry (Chiba et al., 2016; Santos et al., 2018), or indirectly in the case of SP by measuring the amount of internalization of its cognate receptor, neurokinin 1 receptor (NK1) [see (Li et al., 2018; Nazarian et al., 2014) as examples]. The role of SP as a pain-promoting neuropeptide in the spinal cord is well-accepted within the field, and strategies that seek to dampen SP signaling have been sought as potential pain treatments. This is supported by constantly emerging evidence: for example, one study found that deletion of NK1-expressing neurons in the dorsal horn increases pain thresholds in rats in normal and inflammatory pain conditions (Iadarola et al., 2017).

Electrophysiological recordings of spinal dorsal horn neurons are also a method commonly used for predicting pain outcomes, whereby decreased activity of nociceptive neurons correlates with decreased pain sensitivity. Oral co-administration of 5-hydroxytryptamine receptor 3 (5-HT₃) and NK1 antagonists (palonosetron and netupitant, respectively) puts to evidence a synergistic effect of these receptors in the control of mechanical pain thresholds and action potential firing of lamina V/VI dorsal horn neurons

in rats (Greenwood-Van Meerveld et al., 2014). Intraperitoneal (ip) administration of a somatostatin (SST) receptor 4 (SST4) agonist J-2156 was shown to produce antinociception, and decrease the firing rate of primary afferent nerve fibers and mechanosensitive spinal dorsal horn neurons (Schuelert et al., 2015).

Using behavioral assays and combined immunohistochemistry for the cellular activation marker, Fos, Fan and colleagues (2015) showed that intraspinal injection of a corticotropin-releasing factor (CRF) receptor antagonist acting on receptors CRF1 and CRF2 alleviated thermal and mechanical pain in a rat model of bone cancer pain by suppressing neuronal activation in the dorsal horn of the spinal cord. In turn, intrathecal injection of the neuropeptide ghrelin dose-dependently decreased mechanical and thermal allodynia, expression of inflammatory markers [interleukin 1 β (IL-1 β), IL-6, and tumor necrosis factor α (TNF α)] in the dorsal horn of a mouse model of chronic constriction injury (CCI) of the sciatic nerve (Zhou et al., 2014). Activation the neuropeptide Y (NPY) receptor Y1 through intrathecal injection of [Leu31,Pro34]- NPY, alleviates mechanical and cold allodynia in the CCI model (Malet et al., 2017), and decreases SP release from primary afferents onto lamina I neurons in the carrageenan and complete Freund's adjuvant (CFA) models of inflammatory pain (Taylor et al., 2014).

The trigeminal ganglion provides sensory innervation of the face and mouth, hence this specific bundle of nerve fibers being the target of models of orofacial pain. It has recently been shown that after infraorbital nerve injury, NPY expression is upregulated in the trigeminal ganglia (Lynds et al., 2017), and the same can be observed after CCI of the mental nerve (Magnussen et al., 2015).

Brain processing of nociceptive transmission is a rather complex phenomenon: a multitude of brain areas work collectively to evaluate the perceived stimulus for a variety of factors, including its location in the body, the type of pain, and the discomfort it

produces. As such, when studying the role of a specific neuropeptide system on nociception, preclinical studies usually target a single brain area to dissect the functions that that area and neuropeptide system have in pain processing.

A test commonly used to distinguish between acute or more persistent actions of a given treatment is the formalin test. In this paradigm, the animal is given an injection of the inflammatory compound formalin which will produce an initial (“acute”) phase of nociception, due to direct activation of peripheral nociceptors. After 5-10 minutes, pain thresholds will climb again, during what is called the “interphase”, which lasts between 5-10 minutes. Following this, central sensitization of nociceptive cells will trigger the last phase of this test, termed the “tonic” phase, which can last a few hours. This tool allows researchers to discern whether treatments that seek to alter pain thresholds act at a peripheral (acute phase) or central (tonic phase) level by measuring the number of nocifensive behaviors (biting, licking) displayed by the mice. In the rat striatum, SP release and NK1 internalization are increased after injection of formalin in the contralateral paw (Nakamura et al., 2013). In the same study, the researchers found that continuous, but not single, injection of SP into the contralateral striatum decreases nocifensive behaviors in the tonic phase of the formalin test, increases paw withdrawal thresholds, and induces NK1 internalization, without affecting hindpaw edema. In turn, intracerebroventricular (icv) of neuropeptide S (NPS) was found to decrease formalin-induced licking and increases (Peng et al., 2010). Another group found that injection of the neuropeptide orexin-A in the nucleus paragigantocellularis lateralis induces antinociception in the acute and tonic phase of the formalin test (Erami et al., 2012). To investigate the therapeutic potential of single and dual orexin receptor antagonists (SORA/DORA) for pain treatment, McDonald and colleagues (2016) administered a

series of these compounds orally to mice, and found that DORAs produce antinociception in both phases of the formalin test.

To investigate the hedonic value of given stimuli, researchers often resort to the condition place avoidance (CPA/CP) behavioral paradigm. In this test, the animals are repeatedly exposed to a specific context while simultaneously being exposed to a stimulus (drug, footshock, etc.). In a test session, the animal will have the choice between the compartment where it received the stimulus, or an innocuous compartment where no stimulation occurred. The choice to avoid or seek the compartment where the stimulus was provided is taken to mean that the stimulus produces negative or positive emotion, respectively. Two separate studies from the same group provide an example of the use of this test in studying the role neuropeptide signaling in the brain and negative emotion. In a first study, Ide and colleagues (2013) showed that injection of CRF in the dorsolateral part of the bed nucleus of the stria terminalis (dBNST) exacerbates formalin-induced CPA. Following this, the same group showed that pain- and CRF-induced CPA are abolished by intra-dBNST injection of a protein kinase A (PKA) inhibitor (Kaneko et al., 2016), suggesting both forms of CPA require the activation of PKA signaling pathways and likely involve the same mechanisms in the dBNST.

Some studies focus on phenomena resulting from manipulation of entire circuits, and/or resort to transgenic models with systemic changes in neuropeptide signaling. The clearest example of the former is found in studies looking into the role of SST-expressing interneurons. In the dorsal horn of the spinal cord, it was shown that ablation of SST-expressing excitatory interneurons specifically abolishes the expression of static and dynamic mechanical allodynia in inflammatory and neuropathic pain conditions (Duan et al., 2014). More recently, Cichon and colleagues (2017) showed that daily activation, using chemogenetic tools, of SST-expressing inhibitory interneurons of the primary

sensory cortex (S1) prevents expression of mechanical allodynia in a model of neuropathic pain, whereas others have found that optogenetic activation of these interneurons in the anterior cingulate cortex (ACC) does not alter mechanical pain thresholds (Kang et al., 2015). While these studies provide important insight into the hodological composition of circuits that influence pain transmission, they generally fail to identify the specific neurochemicals mediating such effects.

One example of a study using a transgenic model with large-scale changes in neuropeptide signaling is a recent investigation on the role of neurotensin (NTS) receptor 3 (NTS3) on NTS-mediated analgesia. In here, the authors used a transgenic NTS3 deficient mouse model (NTS3^{-/-}) to investigate the effects of this receptor on NTS-elicited anti-allodynia, and found that expression of NTS3 is required for NTS2-mediated anti-allodynia in normal animals (Devader et al., 2016).

The reader is also referred to other reviews considering the role of non-opioid neuropeptides in nociception that are less studied, or have not been the focus of recent literature, including neuropeptides B and W (Dvorakova, 2018), and neuromedins U, B and N (Gajjar and Patel, 2017).

The rapid progress of technical tools for investigating cellular, molecular, and hodological underpinnings of nociception has allowed for the advancement of the understanding of how various systems influence the perception of sensory-discriminative and aversive aspects of pain. This subject remains at the core of the identification of therapeutic targets for pain management.

3. Effects on pain-related behaviors

The most considerable predicament concerning pain management is long-term management of a painful disease/syndrome. Long-term pain leads to maladaptive changes

in neural architecture and synaptic plasticity, which in turn contribute to the expression of comorbid disorders, such as anxiety, depression, and sleep disorders, as well as alterations in the reward system. Since the turn of the century, interest has been growing in identifying the mechanisms through which altered pain sensation can lead to the development of comorbidities, and how the progression of these comorbid states can be halted. Furthermore, current available treatments prescribed to patients suffering from chronic pain target nociceptive signaling, and often disregard the comorbid states that accompany long-lasting pain. These treatments per se also carry significant side-effects (see Section 4 – Relation to opioid signaling), making them inadvisable for continuous use.

It then becomes evident that there is a need to investigate how new candidate therapies affect not only nociceptive transmission, but also other types of behaviors commonly associated with painful syndromes.

3.1 Stress – anxiety, depression, fear and PTSD

Perhaps the most widely studied pain comorbidity is its relation to stress. On a temporal scale, stress can be divided into acute and chronic according to its duration. Acute stress comprises the activation of the paraventricular nucleus of the hypothalamus (PVN) and the release of CRF onto the pituitary gland, which in turn releases adrenocorticotrophic hormone (ACTH) onto the adrenal glands, which release cortisol (in humans) or corticosterone (CORT, in rodents) (Lariviere and Melzack, 2000). CORT then acts as a negative feedback modulator of this circuit, termed the hypothalamic-pituitary-adrenal (HPA) axis. Several neuropeptides have been reported to affect HPA axis activity at different levels, thus influencing stress responsiveness, including galanin, oxytocin, arginine vasopressin, NPS, NPY, pituitary adenylate cyclase-activating peptide

(PACAP), CRF, substance P, orexin, somatostatin, and NPY [for reviews see (Kormos and Gaszner, 2013; Sargin, 2018)]. This subsection focuses on recent work showing that different non-opioid neuropeptides influence stress, stress-induced changes of pain responses, and pain-induced changes of stress responses.

Acute stress is necessary for the survival of organisms and is known to interact with nociception by reducing pain sensation, a phenomenon known as stress-induced analgesia (SIA) (Butler and Finn, 2009). One paradigm commonly used to induce acute stress in rodents is exposure to warm or cold water, which rodents prefer to avoid. In this sense, both forms of stress induce analgesia, albeit through different mechanisms, with warm water swim SIA involving activation of opioid receptors, whilst cold water swim SIA requires activation of N-methyl-D-aspartate receptors (NMDARs) (Marek et al., 1992). Indeed, icv injection of NPY decreases warm but not cold water swim SIA, reinforcing the view that NPY actions oppose opioid-mediated behaviors (Li et al., 2012) (for further detail see Section 4 – Relation to opioid signaling). In rats exposed to water avoidance stress, blocking CRF1 in the anterolateral part of the BNST (alBNST) decreases an anxiety-like phenotype and mechanical pain thresholds (Tran et al., 2014). In the central amygdala (CeA), both sustained inhibition of CRF-expressing neurons, as well as ablation of CRF-expressing neurons projecting to the locus coeruleus (LC), restores forced swim-induced SIA and increases mechanical pain thresholds in a mouse model of neuropathic pain (Andreoli et al., 2017). In keeping with this, CeA CRF knockdown was shown to rescue decreased visceral and somatic pain thresholds after water avoidance stress or colonic distension, another model of acute stress (Johnson et al., 2015).

While acute stress is crucial for survival, chronic stress comprises a series of maladaptive changes, resulting from overstimulation of the HPA axis, that culminate in decreased quality-of-life and increased propensity for developing stress-related disorders,

such as generalized anxiety disorder (GAD), major depressive disorder (MDD), and post-traumatic stress disorder (PTSD). These disorders themselves influence pain thresholds and several pain syndromes affect stress responses. One example of the latter is irritable bowel syndrome, a painful disorder characterized by abdominal pain, altered defecation and stress-induced changes in intestinal motility (Nozu and Okumura, 2015). Both central and peripheral CRF receptors, particularly CRF1, have been investigated as potential therapeutic targets for the treatment of this disorder due not only to their antinociceptive actions, but also their effects on alleviation of anxiety symptoms (Nozu and Okumura, 2015; Taché, 2015). Because of its prominent role in initiating the stress response, CRF has been investigated as a potential target for the treatment of stress-related conditions. In a rat model of monoarthritis, blocking CRF receptors in the LC results in alleviation of pain-induced anxiety-like behaviors, but not pain itself (Borges et al., 2015). In the CeA, disruption of NMDAR GluN1 subunit expression in CRF-positive neurons enhances acquisition and retention of auditory fear conditioning (Gafford et al., 2014). In this same structure, in a predator scent stress (PSS) model of PTSD, blocking CRF1 abolishes a hyperalgesic phenotype (Itoga et al., 2016). Altogether, this evidence points towards CRF receptors being prominent targets in the treatment of stress-related disorders, including pain-induced stress conditions, as well as stress-induced pain syndromes.

On another hand, NPS has also been the focus of a considerable body of research regarding its anxiolytic properties on their own or in the context of painful conditions. In the early 2000s, NPS was first described as neuropeptide that could increase wakefulness and suppress anxiety-like behaviors (Xu et al., 2004). More recently, it has been shown that icv administration of NPS dose-dependently produces panicolytic effects (Pulga et al., 2012), and that intranasal application of NPS reduces anxiety-like behaviors (Ionescu

et al., 2012), and decreases pain sensation and anxiety levels in a rat model of arthritis (Medina et al., 2014). In a CCI model, NPS mRNA and peptide content are reduced in the amygdala, and these findings are concomitant with an anxiety-like phenotype which can be reversed by icv administration of exogenous NPS (Zhang et al., 2014). Moreover, administration of NPS in the medial amygdala (MeA) has also been shown to produce anxiolysis (Grund and Neumann, 2018), while in the basolateral amygdala (BLA) it was shown to alleviate stress responses in the PSS model of PTSD (Hagit et al., 2018).

In sum, evidence supports the involvement of non-opioid neuropeptide systems in stress-related disorders, at various levels of this spectrum. In addition, several of these neuropeptide systems may prove useful targets for new therapies in the treatment of stress-related disorders and comorbid pain syndromes, and vice-versa.

3.2 Feeding and drinking

The idea that food-seeking behavior and/or drinking share a common thread with pain is a fairly new concept. In a recent study, food-deprived mice were shown to have decreased nociceptive responses to inflammatory pain, through NPY signaling from arcuate nucleus (ARC) agouti-related peptide (AgRP)-expressing neurons to Y1 receptors in the parabrachial nucleus (PBN), in a way that did not require the other co-transmitters, GABA and AgRP (Alhadeff et al., 2018). Indeed, other neuropeptide systems that are known to play a role in nociception, are also involved in food-seeking and drinking. NTS is a well-known anorectic peptide (Boules et al., 2013), and a population of NTS-containing lateral hypothalamus neurons was recently identified as being able to drive drinking independently of palatability, with delayed suppression of feeding (Kurt et al., 2018; Woodworth et al., 2017). Additionally, SST-expressing neurons of the tuberal nucleus impinging on the BNST and PVN promote food-seeking behavior (Luo et al., 2018),

which is in line with previous findings supporting a role for somatostatin in feeding as well as drinking (Stengel et al., 2015). Intra-amygdalar injection of RFRP-3, an endogenous ligand for NPF2R, dose-dependently decreases food intake in rats (Kovács et al., 2014).

The aforementioned brain areas and neuropeptide systems have been shown to influence nociceptive transmission. Whether similar mechanisms and cell types are involved in both nociception and feeding/drinking remains to be clarified, as well as the physiological or pathological conditions that need to be met to initiate interplay between these behaviors.

3.3 Addiction – reward and motivation

Addiction and chronic pain interact on two separate levels. Firstly, chronic pain is accompanied by changes in reward circuitry, resulting in both reward deficiency and anti-reward, which exacerbate pain intensity and chronicity (Borsook et al., 2016). Secondly, the continuous use of opioid drugs (see also Section 4 – Relation to opioid signaling) for management of long-term pain, which activate reward pathways, promotes the establishment of drug abuse disorders. Thus, expanding knowledge into how emerging therapies affect reward and motivation in itself and in pain conditions is highly relevant.

Indeed, some neuropeptide systems that are known to play a role in nociception have also been linked to reward-seeking behaviors. It was recently shown that systemic and localized injection of morphine in the ventral tegmental area (VTA) increases release of SP in the VTA (Sandweiss et al., 2018). In turn, intra-VTA administration of SP or morphine increases dopamine (DA) release onto nucleus accumbens (NAc) via NK1 activation, suggesting SP- and morphine-mediated mechanisms for facilitating DA release onto NAc share common pathways (Sandweiss et al., 2018). Additionally, genetic

ablation of NK1 in the VTA prevented expression of morphine-induced conditioned place preference (CPP). Likewise, icv administration of NPS also decreases morphine-induced CPP (Li et al., 2009b). Opposite to this, injection of orexin-A in the dentate gyrus facilitates acquisition, expression, and reinstatement of morphine-induced CPP (Guo et al., 2016).

One neuropeptide that has been more heavily focused in recent literature regarding its effects both on pain and reward-seeking behaviors is CRF. In one study, it was shown that CCI, a model of neuropathic pain, facilitates the expression of morphine-induced CPP through sustained activation of medial prefrontal cortex (mPFC) CRF-expressing neurons projecting to the NAc, and involved activation of CRF1 (Kai et al., 2018). The authors showed that chemogenetic inhibition of mPFC CRF neurons and optogenetic activation of mPFC-NAc CRF neurons prevents the expression of morphine-induced CPP in CCI mice, whereas optogenetic activation of this pathway in sham mice recapitulated the effects of CCI on morphine-induced CPP (Kai et al., 2018). Interestingly, none of these chemo- or optogenetic manipulations resulted in changes in pain thresholds, suggesting this pathway is specifically involved in reward-seeking behavior. Increased CRF and CRF1 mRNA in the CeA is associated with decreased thermal pain thresholds in nicotine-dependent rats, which is reversed by systemic and local blockade of CRF1 (Baiamonte et al., 2014). Systemic CRF1 antagonism also alleviates hyperalgesia in ethanol-dependent rats (Edwards et al., 2012).

Several non-opioid neuropeptides that influence reward-seeking in preclinical models of pain as well as addiction have been focused in recent reviews, including SP (Muñoz and Coveñas, 2014; Sandweiss and Vanderah, 2015), NPY (Thorsell and Mathé, 2017), orexin (Tsujino and Sakurai, 2013), and CRF (Zorrilla et al., 2014). The prominence of this subject in the fields of pain and addiction is evident, and future studies should

consider the interactions between pain and reward circuitry as important players in the expression of nociception as well as conditioned behaviors.

3.4 Sleep and wakefulness

The interactions between sleep and pain have been the focus of several studies for the past decades [see (Lautenbacher et al., 2006) for a review]. More recently, in a large human cohort study, it was shown that several key features of sleep, including efficiency, as well as frequency and severity of insomnia, were associated with pain sensitivity (Sivertsen et al., 2015). Moreover, the authors found a synergistic effect, regarding reduced pain thresholds, in patients reporting both insomnia and chronic pain (Sivertsen et al., 2015).

Indeed, understanding the bidirectional relationship between sleep and pain is crucial. Despite this, the role individual neuropeptide systems play in coordinating sleep and pain remains largely unstudied. In recent years, it was shown that administration of the NPSR antagonist [D-Cys(tBu)⁵]NPS decreased wakefulness and increased time spent in non-rapid eye movement (REM) sleep without affecting REM sleep (Oishi et al., 2014). Additionally, icv administration of NPS attenuated REM sleep deprivation-induced anxiety-like phenotype (Xie et al., 2018). These studies provide important insights into how the NPS/NPSR system affects both sleep and sleep-related behavior changes, with their relation to nociception being outside the scope. Nevertheless, the fact remains that by showing that the NPS/NPSR and other systems, such as orexin [see (Nevárez and de Lecea, 2018) for a review] and Neuropeptide B (Dvorakova, 2018), together with their cognate receptors, influence the sleep/wake cycle, it can be postulated that specific activation/inhibition of these systems can produce alleviation of comorbid sleep and pain disorders.

4. Relation to opioid signaling

Opiate alkaloids isolated from the opium poppy *Papaver somniferum* have been used by humans for thousands of years. In 1975, the first report emerged of two forms of enkephalin, an endogenous opioid receptor agonist, being present in pig brain, and having potent agonist activity at opioid receptors (Hughes et al., 1975). This opened the field to the idea that there was a ligand-receptor system that could be exploited for the purpose of controlling pain thresholds.

Opioid peptides are important effectors of nociceptive signaling that act through μ -, δ -, and κ -opioid receptors (MOR, DOR, and KOR, respectively). Morphine, an agonist for all three ORs, is the “gold standard” against which other analgesics are compared. Even though it is a highly potent painkiller, its use is accompanied by deleterious side-effects that limit its long-term use. These can range from constipation, nausea, and vomiting to addiction, respiratory depression, and death (Schuckit, 2016). As such, one of the goals of preclinical research is to understand how new emerging therapeutic options may affect opioid signaling.

Evidence for interactions between opioid and non-opioid neuropeptide systems is present in studies focusing on molecular, cellular, and/or behavioral components of the nociceptive network. The most notable group of non-opioid neuropeptides with opioid-modulating properties is the RF-amide family of peptides. NPFF, a member of this family, opposes opioid-induced changes in several types of behavior, by decreasing reward-seeking, locomotor activity, feeding and drinking, intestinal motility, and nociception, when co-administered in supraspinal structures [see (Moulédous et al., 2010) for a review], despite having low affinity for opioid receptors (Yang et al., 2008). Other members of the RF-amide peptide family, including RFRP-3, 26RFa, PrRP-20, and Kp-10 have been shown to elicit NPFFR-dependent hyperalgesia in the tail immersion test,

with the latter two partly reversing morphine analgesia via the same receptors (Elhabazi et al., 2013). Additionally, subcutaneous injection of a NPFFR antagonist potentiates the analgesic effects of morphine, and ameliorates naltrexone-precipitated signs of withdrawal in morphine-dependent mice (Elhabazi et al., 2012), suggesting NPFFR blockade ameliorates the side-effects of long-term use of opioid analgesics.

Other non-opioid neuropeptide systems interact with opioid peptides. Recent work has shown that the SST4 receptor forms heterodimeric complexes with DORs in the cortex, striatum and spinal cord, and that simultaneous activation of both receptors leads to decreased cAMP and PKA activation in a cooperative manner (Somvanshi and Kumar, 2014). At the behavioral level, co-administration of orexin-A and morphine into the medial pre-optic area was shown to produce potentiated analgesia, which puts to evidence a synergy between OX1R and opioid receptors (Emam et al., 2016). In line with this, central activation of the ghrelin receptor GHSR1a produces DOR- and KOR-dependent analgesia, and icv administration of morphine and GHSR1a agonist GHRP-2 produce synergistic analgesia (Zeng et al., 2014). In opposition to this, one study reported that ghrelin and GHSR1a agonists attenuate analgesia after i.p. injection of morphine (Zeng et al., 2013), indicating that the signaling phenomena that mediate interaction between opioids and this particular neuropeptide system are complex.

Nevertheless, it should be noted that some non-opioid neuropeptide systems have been shown to produce opioid-independent analgesia when activated. Icv administration of NPS has been shown to produce analgesia even after co-administration of the OR antagonist naloxone (Li et al., 2009a). Furthermore, when initially tested for its antinociceptive properties, orexin-A was shown to produce thermal analgesia even after i.p. injection of naloxone (Bingham et al., 2001).

Regardless of the lack of interaction found in some non-opioid neuropeptide systems, studying the effect of OR signaling in relation to analgesic, anxiolytic, addictive, and other properties of new therapies is of the utmost importance, as it allows researchers to identify cross-talking phenomena between these systems, furthering the progress of identification and development of viable targets/treatments for the treatment of pain.

5. Interactions between non-opioid neuropeptide systems

As previously stated, although opioid drugs are potent painkillers, their use is accompanied by a plethora of undesired side-effects that limits their long-term use. Thus, interest has been increasing on the discovery of other molecules that can produce analgesia in the clinical setting, with more limited side-effects. In this regard, researchers have been looking into how non-opioid neuropeptides, which share signaling pathways with ORs, work in tandem to fine-tune nociceptive responses. Understanding how interplay between different systems manipulates pain sensation has become pivotal for the development of therapeutic strategies.

In the spinal cord, SP and CGRP, together with the classical neurotransmitter glutamate, help regulate nocifensive responses in the tonic phase of the formalin test (Rogoz et al., 2014). Glutamate and SP, and glutamate and CGRP, released from primary afferent neurons of the DRGs, help mediate cold and heat sensation, respectively (Rogoz et al., 2014), suggesting interactions between individual neuropeptides and classical neurotransmitter systems are also relevant for maintaining normal pain thresholds. Additionally, in inflammatory pain conditions, SP release from primary afferents onto layer I neurons of the dorsal horn is decreased by intrathecal injection of [Leu31,Pro34]-NPY, resulting in activation of Y1 receptors (Taylor et al., 2014). On the other hand, CGRP expression in the spinal cord and DRG, usually associated with increased pain

sensation, has been shown to be increased in a mouse model of NPFFR2 overexpression, or after administration of a NPFFR2 agonist (Lin et al., 2017a), reflecting that the actions of RF-amide family peptides go beyond those of simply modulating opioid receptor activity.

In the dorsolateral part of the BNST (dlBNST), CRF-induced CPA is attenuated by coadministration of NPY in a Y1 and Y5 receptor-dependent manner (Ide et al., 2013). Parallel to this, ghrelin has been shown to depolarize AgRP/NPY neurons of the ARC through activation of its cognate receptor GHSR1a (Chen et al., 2017), and, as stated above (Section 3.2 – Feeding and drinking), NPY signaling from the ARC to the PBN mediates hunger-elicited pain hyposensitivity (Alhadeff et al., 2018), which may in part explain the findings of Alhadeff and colleagues, as ghrelin is released into the bloodstream to induce hunger (Sato et al., 2012).

The interactions between different neuropeptide systems are of interest for the understanding of pain biology, as well as other related behaviors. In the PVN, which is densely innervated by pericoerulear NPS-containing fibers, silencing of PVN oxytocin-positive neurons blocks anxiolytic actions of icv administered NPS (Grund et al., 2017), and these neurons have been shown to also play a role in nociception (Eliava et al., 2016). In this nucleus, activation of NPFFR2 increases serum corticosteroids in a CRFR-dependent manner, leading to cell activation in the PVN, and increasing anxiety-like behaviors (Lin et al., 2017b). Additionally, in a model of PTSD, the anxiolytic effects of NPS administration in the BLA were shown to be dependent on activation of NPY Y1 receptor (Hagit et al., 2018).

From a biological standpoint, it is noteworthy that no single manipulation of a receptor system results in an isolated effect. The inner workings of organisms are those of cogs

and gears, interconnected to ensure survival. As such, gaining insight into how the different components are linked and work together is of the utmost importance.

6. Interspecies differences and sexual dimorphisms

Despite the development of numerous candidate molecules targeting non-opioid neuropeptide systems for the management of pain (Pérez de Vega et al., 2018), few reach the clinical trial stage and even fewer prove useful in the clinical setting. Two major hindrances gate the transition of candidate drugs into viable pharmacological treatments. Firstly, preclinical studies often resort to the use of non-human mammals to explore how manipulations of neuropeptide systems influence nociception to measure nocifensive behaviors or estimate nociceptive thresholds in intact organisms. The difficulty then lies with how organisms from different species react to the same manipulations. Occasionally, reports emerge containing findings that differ from previous studies, and these differences are often attributed to the use of a species different from the one previously used.

Boules and colleagues (2013) reviewed evidence concerning the effects of NT receptor agonists in a variety of behaviors, including pain. In this review, the authors stated that “evidence suggests that the analgesic efficacy of NT analogs varies with their selectivity for NTS1 and NTS2, the pain model, and, probably, animal species”. We would agree with this point, given the evidence presented by the authors, and would expand it to other non-opioid neuropeptide systems. For example, one study has shown that pharmacological activation of NPPFR2 or inhibition of NPPFR1 produce analgesia in Sprague-Dawley (SD) rats (Lameh et al., 2010), while another study using Kunming mice found that neither activation or blockade of each of the two receptors produced changes in nociception (Fang et al., 2011). Additionally, other members of the RF-amide peptide family, such as RFRP-3, Kp-10, PrRP-20, and 26 RFa, have been shown to induce

NPFFR-mediated hyperalgesia in C57Bl6/N mice in the tail immersion test (Elhabazi et al., 2013). While the authors in these studies used different compounds and routes of administration (Lameh and colleagues used i.p. administration of non-peptidomimetic analogues that can cross the blood-brain barrier (BBB), and Fang and colleagues, as well as Elhabazi and colleagues, administered peptidic compounds icv), it is noteworthy that the expression profile for the two receptors varies among different rodent species (Gouardères et al., 2004), which may partly account for the observed differences.

Moreover, in a model of pristane-induced arthritis, intraplantar administration of the SST analog octreotide produces mechanical analgesia, and decreases C fiber firing in DA, but not DA.1U rats (Yao et al., 2016), and it had been previously shown that these two strains have different levels of pain sensitivity (Guo et al., 2015). The sole difference between these two strains lies in the presence of different alleles for the major histocompatibility (MHC) (Stevenson et al., 1997), and yet they present clearly distinct responses to nociceptive stimulation. As such, it becomes expectable that more evolutionarily distinct species can display even bigger differences.

Another aspect that limits the validation of proper therapeutic targets that can be used in the clinical setting is that most preclinical studies opt for the use of male over female animals. The differences in nociception between the two sexes are well documented, and of great interest in the field, as the underlying mechanisms are still poorly understood (Mogil and Bailey, 2010). However, the variability of pain thresholds in females depending on their oestrus cycle, which leads to changes in hormone levels, adds a layer of complexity to study design that can be hard to circumvent. Notwithstanding, there are reports that document differences and similarities between males and females when a neuropeptide system is activated and/or inhibited.

One report found that male and female VIP^{-/-} mice display increased microglial activation in the dorsal horn of the spinal cord and decreased bilateral paw withdrawal thresholds following unilateral spared nerve injury (Gallo et al., 2017). Additionally, the authors showed that these mice develop bilateral sensitization after hindpaw incision, which persists for longer than unilateral sensitization observed in VIP^{+/+} (Gallo et al., 2017). This evidence suggests that VIP plays a role in lateralization and chronification of pain, and stands out as an example of a scenario where males and females exhibit similar responses to similar manipulations.

In opposition to this, analysis of nocifensive behaviors in the formalin test showed differences between male and female NTS1^{-/-} mice in regard to dose-response relationships after morphine administration (Roussy et al., 2010), suggesting there is sexual dimorphism in functional interaction of the neurotensinergic and opioidergic systems. In the tonic phase of the formalin test, SP release in layer III/IV and V/VI neurons of the dorsal horn of the spinal cord, measured as NK1 internalization, is higher in female than male rats (Nazarian et al., 2014). In the same study, it was shown that ovariectomy reduced SP release, which was reinstated by administration of estradiol in ovariectomized rats, and that gonadectomized male and female rats had similar levels of SP release after formalin injection (Nazarian et al., 2014). Furthermore, injection of the SST analog octreotide in the ventrolateral orbital cortex inhibits tail flick reflex and formalin-induced nocifensive behaviors in female but not male rats (Qu et al., 2015). This puts to evidence the well-known fact that males and females respond differently to pain, more specifically in the case of manipulations of non-opioid neuropeptide systems.

Moreover, the expression of other behaviors that can affect pain thresholds, such as anxiety, can also differ between males and females. For example, the maternal separation (MS) model of anxiety has been shown to not produce changes in anxiety-like behaviors

in male mice (Tan et al., 2017), while it produced changes in anxiety-like behaviors in female mice of the same species (Pierce et al., 2014). Additionally, MS females displayed vaginal hypersensitivity (Pierce et al., 2014), which represents a clear example of how anxiety and pain are connected and are differently modulated in males and females.

These lines of evidence highlight the need for preclinical studies to consider the use of different species, albeit the need to replace animals for simpler models remains imperative. Furthermore, expanding the scope of studies to include females will allow researchers to more easily identify biased and non-biased targets for management of pain.

7. Development of pharmacological tools

Ultimately, the goal of these investigations into non-opioid neuropeptide signaling is to develop new therapeutic options for short- and long-term pain management. These treatments should preferably dampen nociceptive signaling and the expression of comorbid conditions, prove safe for clinical use (reduced toxicity), have limited side-effects, and be of simple administration, with a reduced number of administrations required to achieve therapeutic concentrations, thus having good pharmacokinetic and pharmacodynamic profiles.

In this sense, studying non-opioid systems has allowed for the development of specific ligands and small molecule agonists/antagonists, some of which can cross the BBB. Recently, Elhabazi and colleagues (2017) showed that in rats oral administration of RF313, a NPPFR antagonist with no apparent side-effects first identified in 2015 (Bihel et al., 2015), potentiated morphine analgesia, whilst attenuating opioid-induced hyperalgesia (OIH) and tolerance. In turn, Demeule and co-workers (2014) combined the brain-penetrant properties of Angiopep-2 (An2) with NTS, thus developing the conjugate ANG2002, which they administered peripherally to produce analgesia in models of

inflammatory, neuropathic, and bone cancer pain. Additionally, like neurotensin, this compound proved to be more potent than morphine at equimolar doses. Another study focusing on the signaling properties of NK1 showed that NK1 internalization to endosomes is required for sustained activation of dorsal horn lamina I neurons, and expression of allodynia and nocifensive behaviors in inflammatory pain conditions (Jensen et al., 2017). In this study, the authors showed that conjugation of NK1 antagonists, namely spantide I (Span), with cholesterol (Chol) dampened endosomal NK1 signaling and produced analgesia in mice. Furthermore, Span-Chol was shown to be stable in human cerebrospinal fluid (CSF).

Efforts to combine the analgesic properties of opioids with those of other neuropeptides have also been the focus of some studies. The opioid-neurotensin hybrid peptide PK20, as well as its metabolite PK20M, has been shown to produce thermal analgesia when administered both centrally and peripherally (Kleczkowska et al., 2013, 2010). Two other studies assessed the analgesic properties of several OR agonist/NK1 antagonist in the CCI model of neuropathic pain upon intrathecal administration (Guillemyn et al., 2015; Starnowska et al., 2017). In 2011, Yamamoto and colleagues first described TY032, a DOR and MOR receptor agonist and NK1 antagonist (Yamamoto et al., 2011). This compound was later used by the same laboratories, and shown to produce analgesia in a neuropathic pain model, without inducing CPP (Sandweiss et al., 2018). The latter observation stands as a relevant point for modern drug development when considering the use of opioid/non-opioid neuropeptide hybrids, as currently used opioids have addictive properties and produce CPP in preclinical animal models. In line with this, the CRF1 antagonist E2508 was shown to alleviate visceral pain and reduce restrain stress-induced defecation without altering basal bowel patterns (Taguchi et al., 2017).

Thus, it is evident that the relevance of understanding how new compounds affect both pain and related behaviors is ever growing. As research in this field progresses, we would expect the focus to gradually shift away from a nociception-centric perspective to a more comprehensive one, where pain stands at the center of a dense network of intertwined behaviors and comorbid disorders.

Moreover, we would refer the reader to reviews on recent progress in the development of new treatments targeting non-opioid neuropeptide systems (Pérez de Vega et al., 2018), particularly SP (Hallberg and Sandstrom, 2018; Muñoz and Coveñas, 2014) and CRF (Zorrilla and Koob, 2010), as well as studies focusing on the development of ligands for the bradykinin receptor 2 (BK2) (Deekonda et al., 2015; Lee et al., 2015, 2014).

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**Chapter 3 – Modulation of inflammatory pain
by relaxin-3/RXFP3 signalling in the rat and
mouse brain**

3.1 Introduction

Diverse neuropeptide systems influence nociception through a plethora of mechanisms, including modulation of descending pain pathways (see Chapter 1). My research focused on a neuropeptide/receptor system that had yet to be tested for its involvement in pain perception: the relaxin-3/RXFP3 system. In this Chapter, I will review the evidence that led us to hypothesize this system could abrogate changes in pain thresholds, specifically through modulation of descending pain pathways. Additionally, I will present evidence from my behavioural studies suggesting that modulation of RXFP3 alters pain sensitivity, as well as discuss the implications of that evidence for future studies.

3.1.1 Relaxin-3

Relaxin-3 (RLN3) in rodents and its human equivalent, H3 relaxin, are neuropeptides that belong to the relaxin family of peptides (Bathgate et al., 2013). Since their discovery almost two decades ago (Bathgate et al., 2002), many studies have focused on describing the biochemical properties of RLN3, and the anatomical distribution and functions of the peptide and its receptors in rodent brain. The vast majority of RLN3-containing neurons in the brain are located in the *nucleus incertus* (NI) (Burazin et al., 2002; Smith et al., 2010), although smaller populations of RLN3-expressing neurons have also been described in the PAG, pontine raphe nucleus (PnR), and the area dorsal to the *substantia nigra* (Smith et al., 2010; Tanaka et al., 2005).

There are four relaxin family peptide receptors (RXFP) – RXFP1-4 – and in cell-based systems, RLN3 can bind to and activate three of them – RXFP1, RXFP3 and RXFP4 (Bathgate et al., 2013; Liu et al., 2003a, 2003b). RXFP3 was first identified as an orphan receptor in 2000 (Matsumoto et al., 2000), and has been extensively studied regarding its signalling pathways, its localization in different brain regions, and the wide range of

behaviours affected by its modulation. The first two topics (signalling and function) will be covered below, and the localization data will be detailed in the Chapter 4, where I will expand on the neurochemical and hodological organization of RLN3/RXFP3 networks.

3.1.2 RXFP3 – signalling and function

RXFP3, like other relaxin family receptors, is a G-protein-coupled receptor (GPCR) that is coupled to a $G_{i/o}$ protein (Liu et al., 2003b). RXFP3 activation inhibits adenylyl cyclase (AC) (Liu et al., 2003b) and, through activation of protein kinase C (PKC) (Westhuizen et al., 2007) and phosphoinositide 3-kinase (PI3K) (Bathgate et al., 2013), increases phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) (**Figure 3.1**). Due to its widespread distribution in the brain, RXFP3 exerts various functions according to its location.

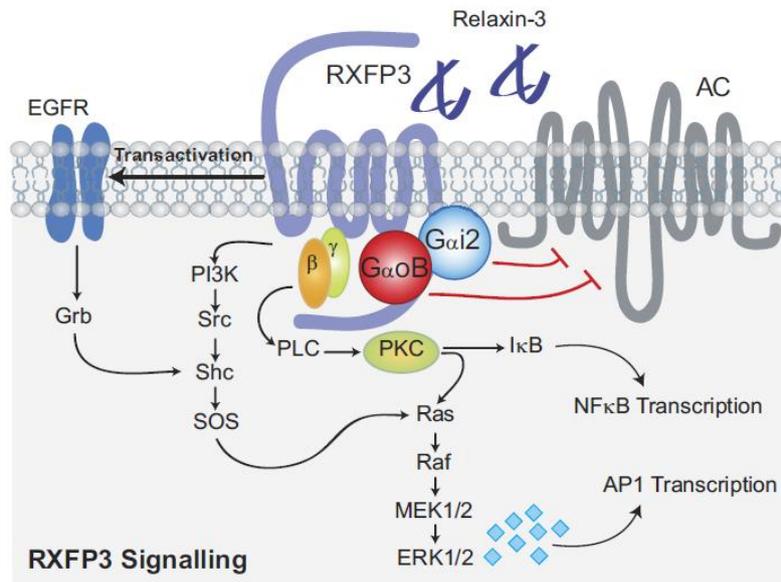


Figure 3.1. RXFP3 signalling pathways. Upon activation of RXFP3, coupling to $G_{\alpha_{i2}}$ and $G_{\alpha_{oB}}$ proteins leads to inhibition of AC. Release of the $\beta\gamma$ subunits activates PI3K and PKC pathways, leading to activation of ERK1/2 and subsequent changes in gene expression, particularly AP1 transcription. Figure and legend adapted from (Bathgate et al., 2013).

Due to the lack of selectivity of H3 relaxin and RLN3 for RXFP3, several RXFP3 agonist and antagonist peptides have been developed to understand receptor pharmacology and involvement in different behavioural modalities. These compounds display distinct binding properties and specificity for different relaxin receptors, but generally have a higher affinity for RXFP3 (Table 3.1).

Table 3.1. Sequences and potencies of relaxin-3-related peptides.

Peptide	Species	Sequence	Estimated potency (pEC ₅₀)		
			RXFP1	RXFP3	RXFP4
Relaxin-3	Rat	RPAPYGVKLCGREFIRAVIFTCGGSRW DVLAGLSSCCCEWGCCKSQISSLC	Slightly higher affinity than H3 relaxin ^a	9.0 ± 0.07 ^b	NE
	Mouse				NE
H3 relaxin	Human	RAAPYGVRLCGREFIRAVIFTCGGSRW DVLAGLSSCCCKWGCCKSEISSLC	8.74 ± 0.06 ^b	9.0 ± 0.07 ^b	8.94 ± 0.13 ^b
		RAAPYGVRLCGREFIRAVIFTCGGSRW CKWGASKSEISSLC	NA ^b	8.43 ± 0.09 ^b	7.7 ± 0.07 ^b
R3(B1-22)R	Synthetic	RAAPYGVRLSREFIRAVIFTSR	NA ^c	7.44 ± 0.05 ^c	NA ^c

NA – no activity; NE – no expression; ^aNo affinity measures were used, but rat/mouse RLN3 displayed similar inhibition of forskolin-induced cAMP accumulation in CHO-K1 cells as compared to H3 relaxin (Hossain et al., 2013); ^bDetermined as ability to stimulate cAMP accumulation (RXFP1) or inhibit forskolin-induced cAMP accumulation (RXFP3 and RXFP4) in HEK-239 cells (Shabanpoor et al., 2012); ^cpK_i values determined using competitive binding assays in comparison to relaxin-3 in RXFP3-expressing HEK-239T (for RXFP1) or CHO (for RXFP3 and RXFP4) cells (Haugaard-Kedström et al., 2011)

RXFP3 activation is known to influence anxiety-like behaviours, as icv injection of the agonist peptide, RXFP3-A2, increased time spent in the light compartment of the light-dark box in rats (Ryan et al., 2013) and mice (Zhang et al., 2015). RXFP3 activation in the PVN inhibited type I OT-expressing neurons (Kania et al., 2017), and OT is a neuropeptide that has been linked to regulation of stress responsiveness and food-seeking behaviours in humans and rodents (Lawson, 2017; Onaka and Takayanagi, 2019), as well as effects on pain thresholds (Gonzalez-Hernandez and Charlet, 2018). Additionally, icv and intra-PVN injection of RLN3 increased plasma ACTH and corticosterone in rats, and H3-relaxin treatment of rat hypothalamus explants increased release of the stress-regulated neuropeptides, CRF and AVP (McGowan et al., 2014). Notably, opposing findings were observed in mice, where icv and intra-PVN administration of RXFP3-A2 did not alter serum corticosterone levels (Zhang et al., 2017).

Interestingly, the relationship between these behaviours and the RLN3/RXFP3 system seems to be reciprocal. Indeed, it has been estimated that approximately 53% RLN3 neurons in the NI express CRF₁ (Ma et al., 2013; Tanaka et al., 2005), and this receptor binds CRF. Exposure to stress paradigms such as water immersion (Tanaka et al., 2005) or restraint stress (Calvez et al., 2015) decreased RLN3 content in the NI. This evidence not only supports the idea that the RLN3/RXFP3 system and stress-regulated behaviours are closely related, but also raises the possibility that other types of stressors (e.g., oxidative stress, fear conditioning, etc.) can induce changes in this system.

One such stressor is food restriction, which has been shown to increase RLN3 mRNA expression in the NI (Lenglos et al., 2013). Like food restriction, restraint stress also enhanced food-seeking behaviour (Lenglos et al., 2013). Likewise, the RLN3/RXFP3 system has been shown to influence food-seeking behaviours. Injection of H3 relaxin in the hypothalamus of rats induced hyperphagia (increased food intake after satiation)

(McGowan et al., 2005). Female rats are thought to be more sensitive than males to the enhancement of food-seeking behaviours by food restriction and stress (Lenglos et al., 2013), and, conversely, to the enhancement of these behaviours by RLN3 (Calvez et al., 2015; Lenglos et al., 2015). Icv administration of RLN3 also increased water intake in rats (Otsubo et al., 2010), whereas the icv administration of the RXFP3 antagonist peptide, R3(B1-22)R, decreased sodium intake in mice (Smith et al., 2015), indicating this neuropeptide system plays a role in regulating osmotic balance. As seen for anxiety-like behaviours, food-seeking also shares reciprocal functioning with the RLN3/RXFP3 system, although the same remains to be verified for osmotic balance-related behaviours. Another function of the RLN3/RXFP3 system is regulation of reproductive behaviour. Icv and intra-paraventricular nucleus injection of H3-relaxin also modulated the hypothalamic-pituitary-gonadal (HPG) axis by increasing the release of luteinizing hormone (LH) and testosterone in a manner dependent on gonadotropin-releasing hormone (GnRH) (McGowan et al., 2008).

The RLN3/RXFP3 system may also modulate arousal and short-term memory. In the NI, RLN3-expressing neuron activity is synchronous with hippocampal rhythm (Ma et al., 2013), which is present during REM sleep, locomotion, and spatial navigation. In line with this, intra-NI injection of lidocaine, a voltage-gated Na⁺ channel blocker, impaired retrieval of spatial memory in the Morris water maze test (Nategh et al., 2015). Evidence exists that RXFP3 may promote arousal, as RLN3 knockout (RLN3 KO) (Smith et al., 2012) and RXFP3 KO mice (Hosken et al., 2015) display reduced activity in voluntary running wheels during the dark phase, when wildtype mice are most active.

Furthermore, the RLN3/RXFP3 system exerts specific control of motivated behaviours, and widely studied addictive behaviours. In the rat NI, RLN3 mRNA expression levels correlate with alcohol and sucrose intake (Ryan et al., 2014), and

RXFP3 and CRF₁ mRNA levels are increased after chronic consumption of alcohol (Walker et al., 2017a). RXFP3 KO mice displayed decreased preference for alcohol consumption after exposure to swim stress (Walker et al., 2015), which is in line with findings in wildtype rats where injection of R3(B1-22)R into the BNST decreased alcohol self-administration (Ryan et al., 2013). Likewise, intra-CeA injection of the RXFP3 peptide antagonist attenuated yohimbine-induced reinstatement of alcohol-seeking behaviour (Walker et al., 2017b).

Indeed, targeting RXFP3 has been proposed as a potential therapy for neuropsychiatric disorders (Smith et al., 2014). Adding to this, the RLN3/RXFP3 system modulates several functions, including motivation, arousal, food-seeking, and anxiety-like behaviours, that are known to be modulated by other neuropeptide systems that influence nociception and are altered in chronic pain conditions. Thus, this evidence is consistent with the idea that modulation of RXFP3 could alleviate pain symptomology by targeting descending controls and mitigating pain comorbidities. Initial studies in our laboratory examined the effect of injections into the lateral cerebral ventricle (LV) of the RXFP3 agonist peptide, RXFP3-A2, in rats that had been injected in their hindpaw with saline 0.9% (NaCl) or complete Freund's adjuvant (CFA), which induces inflammation (**Figure 3.2**; K. Egron and S. Ma, unpublished data). These experiments revealed that activation of RXFP3 produces mechanical analgesia in the von Frey test under normal and inflammatory pain conditions in rats.

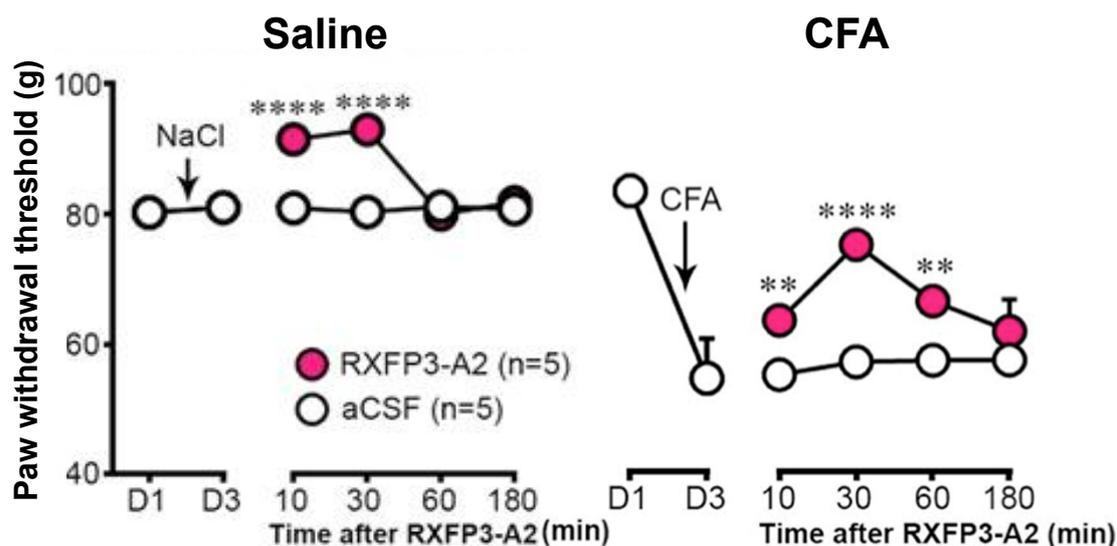


Figure 3.2. RXFP3 activation produces analgesia in normal and inflammatory pain conditions in rats.

Rats were tested for mechanical sensitivity in the von Frey test (expressed as paw withdrawal threshold, in g) before (D1) being injected with NaCl (left graph) or CFA (right graph) in their right hindpaw and re-tested 2 days after (D3). As expected, CFA produced a decrease in paw withdrawal threshold. After this, microinfusions were performed and the rats were submitted to further testing at different timepoints after injection (10, 30, 60 and 180 min) to assess the effect of treatments on nociception. Icv administration of aCSF (white circles) did not alter pain sensitivity. However, microinfusion of RXFP3-A2 (pink circles) produced an increase in pain thresholds in both saline and CFA conditions, indicating central RXFP3 activation produces mechanical analgesia in rats under normal and inflammatory pain conditions. Credit: K. Egron and S. Ma, unpublished data.

3.1.3 Specific Aims

The studies described in this Chapter were aimed at obtaining further evidence for the involvement of the RLN3/RXFP3 system in nociception, and employed targeted intracerebral drug injections in a pain model of persistent inflammatory pain in rats and mice.

3.2 Methods

3.2.1 Animals

For pharmacological studies in mice, experimental procedures followed the ethical guidelines of the International Association for the Study of Pain and were approved by the local ethics committee (University of Bordeaux agreements APAFIS 3654 and APAFIS 13516). Experiments were performed on 7-11 week-old, male C57Bl/J mice.

For studies on rats and RXFP3 KO mice, experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. All experiments were approved by the Animal Ethics Committee at The Florey Institute of Neuroscience and Mental Health (Ethics numbers: 13-104 and 16-092). Experiments were performed using 8-13 week-old, male and female C57Bl6/J RXFP3 KO mice. Sprague-Dawley rats weighed 250-270g upon arrival to the animal facility.

All animals had an acclimation period of 3-7 days upon arrival in the animal facility. Mice were group-housed in groups of 3-6 mice, with males and females kept in separate cages. Rats were single-housed. All animals were handled for at least 2 consecutive days after surgery (where applicable) before the start of experiments. Handling consisted of picking up the animals (cup-hand technique for mice), allowing them to freely move on hands (mice), and then wrapping them in a paper (mice) or cloth towel (rats) and gently restraining them. An exemplary summary and timeline of experimental procedures performed on cannulated animals is illustrated in **Figures 3.3** and **3.4**.

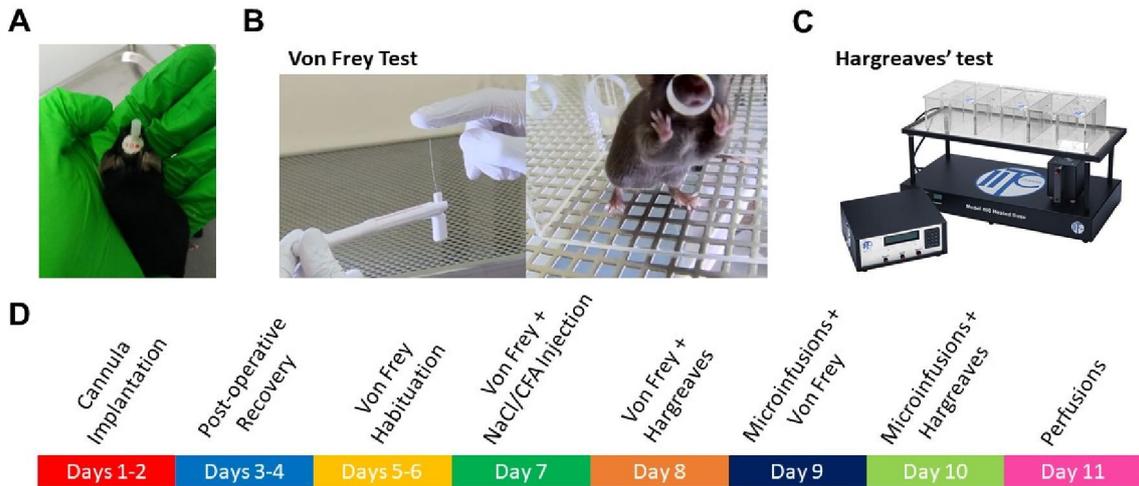


Figure 3.3. Experimental features of behavioural testing in mice. (A) Example of a mouse equipped with a guide and dummy cannulae targeting the LV. (B) Von Frey hair (left) and application of the filament to the plantar surface of the mouse hindpaw (right) to measure mechanical pain thresholds (adapted from (Yalcin et al., 2014)). (C) Hargreaves' device used for thermal pain assessment (image from IITC catalogue). (D) Experimental planning where von Frey and Hargreaves tests were used on the same subject.

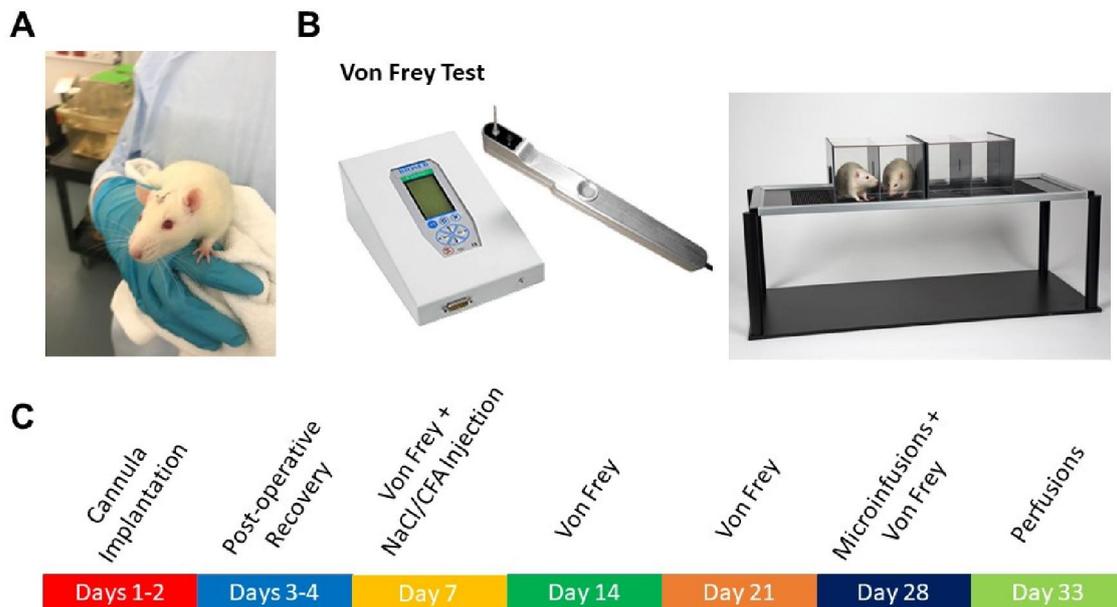


Figure 3.4. Experimental features of behavioural testing in rats. (A) Example of a rat equipped with a guide cannula targeting the LV. (B) Von Frey aesthesiometer (left) and wire mesh and cages where rats were placed to perform the test (right) (adapted from Bioseb Catalogue). (C) Experimental planning used for pharmacological studies in rats.

3.2.2 Cannula implantation surgery

Mice were put under deep anaesthesia using isoflurane 3-5% v/v and placed in a stereotaxic frame, where anaesthesia was maintained at 1.5-2% v/v. The fur was cleaned with ethanol 70% and buprenorphine was administered subcutaneously (s.c.). The fur and scalp were removed, and the surface of the skull was cleaned with green and red activator reagents (Sun Medical, Shiga, Japan). Stereotaxic coordinates were marked using a micropipette filled with Chicago Sky Blue 1% (Sigma-Aldrich) following the coordinates on Paxinos and Franklin (Paxinos and Franklin, 2004) (in mm): for the right LV, anteroposterior (AP) +0.4, mediolateral (ML) -0.75, dorsoventral (DV) -2.7; for the ACC, AP +0.5, ML \pm 0.3, DV -2. One or two burr holes (for LV or ACC, respectively) were drilled targeting these coordinates, and the guide cannula (PlasticsOne, Roanoke, VA, USA) was slowly inserted, which sat at 1.5 mm below the skull surface. The guide cannula was kept in place using Superbond C&B (Sun Medical, Shiga, Japan), which enclosed the entire surgical field, and covered with a dummy cannula (PlasticsOne, Roanoke, VA, USA). The mouse was then assigned a number and put in a recovery cage with a heating pad (32°C) where it stayed until awakening. The mice were monitored over the next 3 days and paracetamol was added to the drinking water to diminish post-operative pain.

Surgical procedures for rats were similar, but with minor modifications. Upon being placed in the stereotaxic frame, the fur over the scalp was shaved and the surgical area was cleaned with ethanol 70% and iodopovidone. Each rat was administered meloxicam intraperitoneally (i.p.) and bupivacaine s.c. before the scalp was incised using a sterile scalpel blade. The skin was pulled aside using surgical clamps. Stereotaxic coordinates used to target the right LV (in mm): AP +0.5, ML -1, DV -4. A burr hole was drilled and the guide cannula (Sun Medical, Shiga, Japan) was inserted, sitting 2.5 mm below the

skull surface. The cannula was kept in place using dental cement (Vertex–Dental, Zeist, The Netherlands). The wound was closed with an interrupted suture and the scalp was cleaned using ethanol 70% and iodopovidone. The rat was left to recover in a heated cage (32°C) and returned to its home cage. Rats were monitored closely until the start of experiments 5-7 days later and were administered meloxicam i.p. daily for the 3 days after surgery to mitigate post-operative pain.

All animals were weighed before surgery and in the 3 days following surgery. If a weight loss of 20% was observed, the animal would have been culled to limit suffering, but this endpoint was never reached in these studies.

3.2.3 Peptides and drugs

Artificial cerebrospinal fluid (aCSF) was prepared to achieve the following composition (in mM): NaCl 130.5, KCl 2.4, CaCl₂ 2.4, NaHCO₃ 19.5, MgSO₄ 1.3, KH₂PO₄ 1.2, HEPES 1.25, Glucose 10, pH 7.4.

RXFP3-A2 and R3(B1-22)R were synthesized using solid phase peptide synthesis and purified using reverse phase high-performance liquid chromatography (HPLC), as described (Haugaard-Kedström et al., 2011; Shabanpoor et al., 2012). Amino acid composition and purity were checked by reverse phase HPLC and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. Peptide stock solutions were prepared by diluting the peptides in aCSF, and made up to the same mass and molar dose by adjustment for purity, which ranged from 85 to 100%. CFA was obtained from Sigma-Aldrich.

3.2.4 Saline and CFA injections

Animals were briefly anaesthetized with isoflurane 4-5% v/v and mounted on a stereotaxic frame or breathing mask, where anaesthesia was maintained at 2% v/v. The plantar surface was cleaned with ethanol 70% and intraplantar injections of sterile saline 0.9% or CFA were performed using a 30-gauge needle connected to a 10 mL syringe. The syringe was kept in place for 20-40 s after injection to prevent any spillage. The animals were then returned to their home cages.

3.2.5 Von Frey test in mice

Mice were placed on a wire mesh one-at-a-time, inside a 5 × 10 cm (for pharmacological studies) or 10 × 10 cm (studies in RXFP3 KO mice) Plexiglas container. Mice were allowed to move freely inside the container to allow habituation to the test environment. Thereafter, 5 von Frey filaments (Bioseb, France), with thickness ranging from 0.4-8 g, were applied 5 times to each hindpaw in random order. For measurement of paw withdrawal threshold (PWT), von Frey filaments (0.4, 0.6, 1 g, 1.4 g, 2 g, 4 g, 6 g) were applied 5 times in ascending order. If the mouse did not respond to a 2-second application of the filament, it was deemed as unresponsive. A response was deemed positive if the mouse withdrew, fluttered, or vigorously licked the paw. When a mouse responded to a filament at least 3 out of the 5 applications, the weight corresponding to the maximum force applicable to that filament was taken as the PWT for that hindpaw and the test stopped.

3.2.6 Hargreaves' test

Thermal stimulation-induced paw withdrawal latency (PWL) was measured using a Hargreaves' device (IITC Life Science, Woodland Hills, CA). A beam of infrared light (30

mA) was applied to the mouse hindpaw, which gradually increased the temperature of the plantar surface, and the time taken for the mouse to withdraw, flutter, or vigorously lick the paw was measured. Mice were allowed to explore the testing chamber for 10-40 min before the start of the experiment, to allow for habituation and to limit movement during the test. An upper time limit of 15 s of beam application was set, but was never achieved. A baseline PWL value was measured the day before testing the effect of peptides on PWL. On the test day, mice were first habituated to the chamber before the administration of vehicle/peptide and then returned to the chamber post-injection. In tests involving multiple central injections, mice were also returned to the chamber between injections. Three PWL measurements were made with a two minute interval between them, to avoid tissue damage and false-positive responses. For analysis, the three values obtained for each hindpaw were averaged.

3.2.7 Von Frey test in rats

Rats were placed on top of a wire mesh, inside a Plexiglas cage and PWT was measured by applying a plastic tip connected to a von Frey aesthesiometer (Bioseb) to the plantar surface of each hindpaw with gradually increasing force, until a response was observed (withdrawal, fluttering, or vigorous licking). The mass corresponding to the applied force was taken as a measurement and a total of 5 measurements were performed. The mean of these 5 measurements was taken as the PWT. If a value differed by >40% of the rat's body weight from the average of 5 measurements, it was excluded, and the mean was recalculated.

3.2.8 Intracerebral and intracerebroventricular injections

Injections were performed manually or using a microinfusion pump (Harvard Apparatus 11 Plus, Holliston, MA, USA). Hamilton microsyringes (10 μ L for ACC infusions, 50 μ L for others) were mounted on the pump and connected to polyethylene microtubing, which was connected to the injector cannulae. Animals were gently restrained using a paper (mice) or cloth towel (rats) and injectors were introduced through the guide cannulae. Infusions of 400 nL (ACC) or 2.5 μ L (when injecting two compounds or injecting one compound in rat) were performed over a 1-min period. Infusions of 5 μ L (LV) were performed over 2 min. After each injection, the injector remained in place for 30-60 s to prevent any spillage. The following amounts were used: RXFP3-A2, 1.1 nmol for LV, 0.44 nmol for ACC; and R3(B1-22)R, 1.8 nmol. The experimenter was blinded to the identity of the injected material.

3.2.9 Genotyping

Cohorts of age-matched RXFP3 KO and WT littermate mice were produced from heterozygous pairings and genotypes were identified using a Sigma REExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, Castle Hill, NSW, Australia). Three primers were used for the polymerase chain reaction (PCR): WT forward, 5'-GCTCATAGCAGTGAGG-AAGAAGACG-3'; WT reverse, 5'-GCTGGTTCTCTACCTGATGAAGAGC-3' and; KO reverse, 5'-GGGCCAGCTCATTCCTCCCCTCAT-3'. After an initialisation step (94°C for 2 min), tail samples were exposed to 30 cycles of PCR. Each cycle included a denaturation (94°C for 30 s), annealing [temperatures of 63°C, 62°C and 60°C for 30 s (2 cycles each for the first 6 cycles) and 58°C for 30 s for the remaining 24 cycles] and elongation (72°C for 1 min) phase. Samples were finally exposed to an additional 10 min of 72°C. This PCR yields 232 bp and/or 461 base pairs (bp) products in the presence of

the WT and/or KO alleles, respectively, which were subsequently separated via gel electrophoresis (**Figure 3.5**) and visualised under ultraviolet (UV) light using Invitrogen SYBR safe DNA stain (Life Technologies, Mulgrave, VIC, Australia).

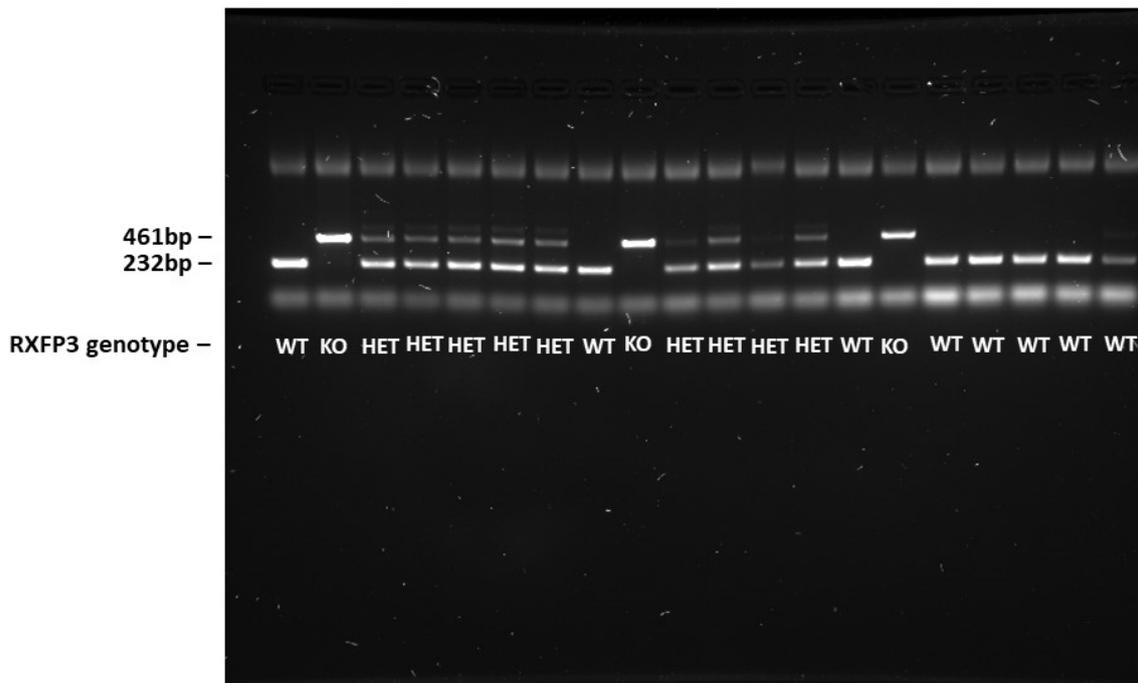


Figure 3.5. Gel electrophoresis of RNA samples of RXFP3 WT and KO mice. PCR products (1 mouse sample per lane) of 232 (WT) and 461 bp (KO) were separated in agarose gel using electrophoresis. The presence of only one product indicates a homozygous genotype, whereas the presence of two products corresponds to a heterozygous genotype. Interpretation of the genotype for each sample in this example is listed under the respective lanes.

3.2.10 Cannula placement verification

After behavioural testing, animals were anaesthetized with lidocaine/pentobarbital (mice) or pentobarbital alone (rats), and transcardially perfused with freshly-prepared 4% paraformaldehyde (PFA). The brain was removed and post-fixed in 4% PFA at 4°C for 1.5-3 h, then placed in a 30% sucrose, 0.1M PBS solution at 4°C overnight (mice) or for 3-7 days (rats). Brains were then frozen on dry ice and kept at -80°C until further processing. For sectioning, brains were mounted in Optimal Cutting Temperature compound (Tissue-Tek® OCT™, Sakura) and 20 µm sections were cut in the cryotome (Leica). For cannula placement verification, sections mounted on a slide (Superfrost Plus, Thermo Fisher Scientific) and examined using light microscopy. Behavioural data from

individual mice or rats was excluded from any further analysis if their guide cannulae and injection sites were incorrect. Examples of correct cannula placements were imaged in an upright epifluorescence microscope (Zeiss Axiophot 2) using a bright-field filter (see **Figures 3.6E, 3.8E, and 3.10E**).

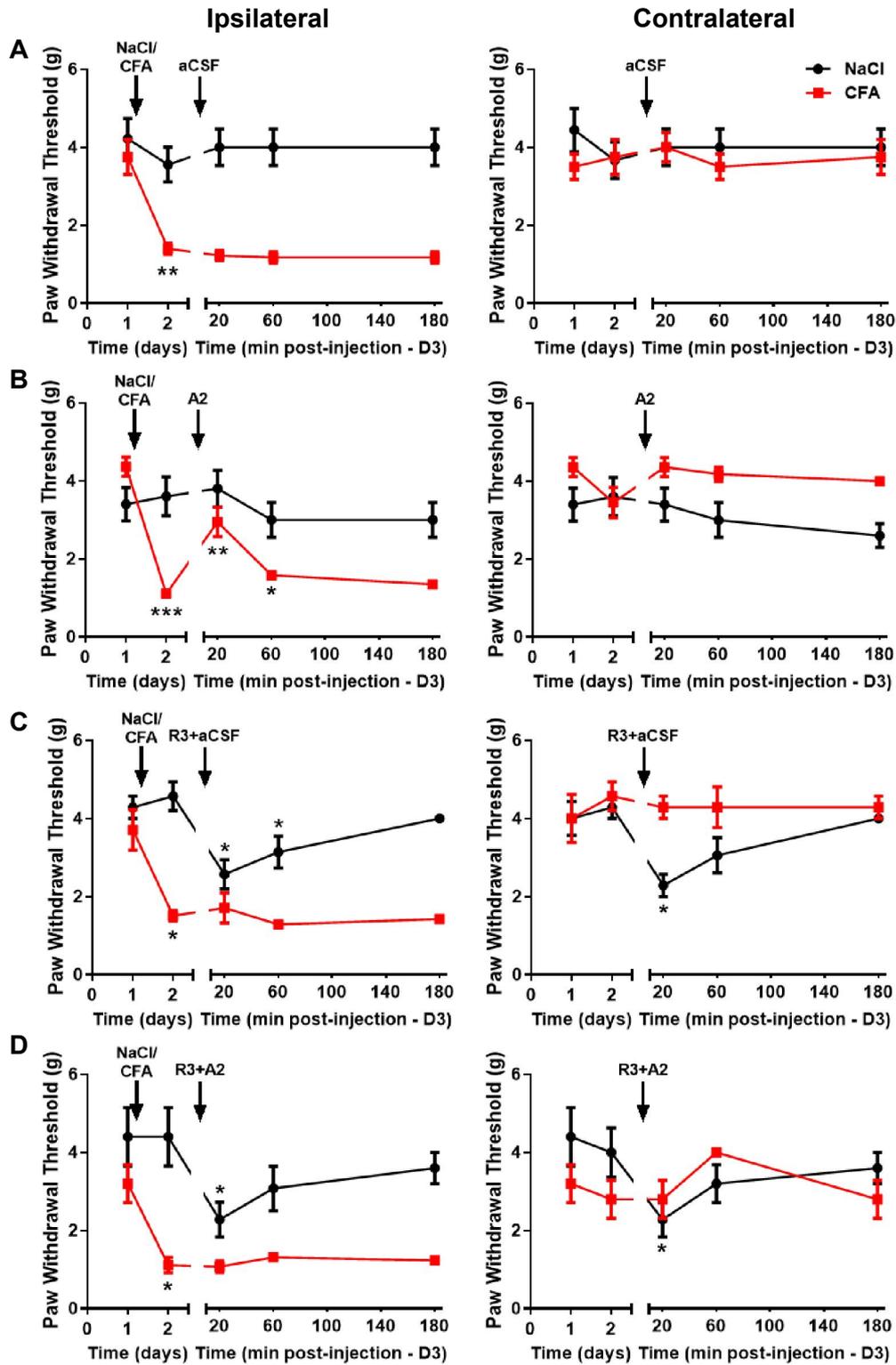
3.2.11 Statistical analysis

Data was analysed using GraphPad Prism 7.0. For PWT measurements, a one-way repeated-measures ANOVA was used to assess the effect of saline or CFA injection, as well as the effect of treatment with aCSF, RXFP3-A2, R3(B1-22)R + aCSF, or R3(B1-22)R + RXFP3-A2, with time as a within factor, followed by Sidak's post-hoc test. The time points compared in mice were: day 1 vs day 2, day 2 vs 20 min, day 2 vs 60 min, and day 2 vs 180 min. Time points compared in rats were: week 0 vs week 1, week 0 vs week 2, week 0 vs week 3, week 3 vs 10 min, week 3 vs 30 min, week 3 vs 60 min, and week 3 vs 180 min. For PWL measurements, a two-way repeated-measures ANOVA was used to assess the effect of saline or CFA injection, as well as the effect of treatment with aCSF, RXFP3-A2, R3(B1-22)R + aCSF, or R3(B1-22)R + RXFP3-A2, with injection as a within factor. ANOVA followed by Bonferroni's multiple comparison post-hoc test was used to assess the interaction between injection and treatment. Data are represented as mean \pm S.E.M. Statistical significance was fixed at $p < 0.05$.

3.3 Results

3.3.1 Pharmacological studies in mice

In these studies I used the von Frey test to assess changes in mechanical pain thresholds in a mouse model of inflammatory pain (**Figure 3.6**). I found that injection of CFA produced a significant decrease in PWT in mice across the separate cohorts ($p < 0.05$). Injection of aCSF did not significantly alter PWT in either control ($n = 9$) or inflammatory pain conditions ($n = 8$) at the time points tested (**Figure 3.6A**). When injected with the RXFP3 agonist peptide, RXFP3-A2, mice injected with CFA displayed an increased PWT 20 min after the injection ($n = 11$, $p < 0.01$) that was attenuated after 1 h, and completely reversed 3 h after injection (**Figure 3.6B**), indicating that RXFP3 activation produced mechanical analgesia under inflammatory pain conditions. Under normal, but not inflammatory pain, conditions, mice injected with the RXFP3 antagonist peptide, R3(B1-22)R and aCSF displayed decreased PWT in both ipsilateral and contralateral ($p < 0.05$) paws ($n = 7$ for both groups, **Figure 3.6C**). Under normal pain conditions, mice injected with the agonist and antagonist peptides displayed a similar behavioural phenotype ($n = 5$, $p < 0.05$, **Figure 3.6D**). In mice injected with CFA, there was no changes in PWT, indicating that the antagonist peptide blocked the analgesic effect of the agonist ($n = 5$, **Figure 3.6D**). An example of a correct cannula placement is illustrated in **Figure 3.6E**.



E

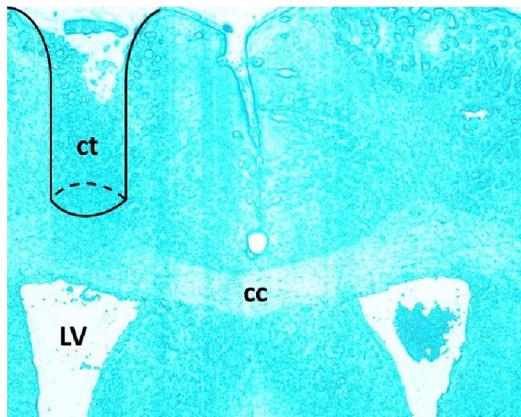


Figure 3.6. Effect of RXFP3 modulation on mechanical pain thresholds. Mice were submitted to von Frey testing to ascertain changes in mechanical pain thresholds, represented as PWT, over several days. Mice that received i.pl. injection with saline (NaCl, black lines and circles) displayed no changes in mechanical pain thresholds, whereas mice injected with CFA (red lines and squares) displayed decreased PWT in the ipsilateral paw in all cohorts when compared to day 1. Injection of saline or CFA is indicated after day 1 by a black arrow and the heading “NaCl” or “CFA”, respectively. Icv injections on day 3 are indicated by a black arrow and the heading indicating the treatment administered [aCSF, RXFP3-A2 (A2), R3(B1-22)R+aCSF (R3+aCSF) or R3(B1-22)R+RXFP3-A2 (R3+A2)]. (A) Mice injected with aCSF displayed no changes in PWT. (B) Mice injected with RXFP3-A2 displayed increased PWT in inflammatory pain conditions 20 min after the injection; this effect was diminished when tested again at 60 min, and completely reversed at 180 min. (C) Under normal but not inflammatory pain conditions, mice injected with R3(B1-22)R and aCSF displayed decreased PWT both at 15 and 60 min after the injection in both hindpaws. (D) Under normal pain conditions, mice injected with R3(B1-22)R and RXFP3-A2 displayed decreased PWT 20 min after the injection. Moreover, R3(B1-22)R prevented the analgesic effect of RXFP3-A2 under inflammatory pain conditions. (E) Example of a cannula tracing targeting the lateral ventricle. cc: corpus callosum; ct: cannula tract; LV: lateral ventricle. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA followed by Sidak’s multiple comparison test for the effect of treatments; day 2 was compared to day 1, and each individual time point on day 3 was compared to day 2.

In studies to assess changes in thermal pain thresholds, I used the Hargreaves’ test. Injection of saline failed to produce changes in PWL, whereas CFA induced systematic decreases in this measure consistent with inflammatory pain-induced thermal allodynia (**Figure 3.7**). Injection of aCSF into the lateral cerebral ventricle produced no changes in thermal pain threshold, as expected (**Figure 3.7A**). Likewise, injection of the RXFP3-A2 agonist did not alter PWL, suggesting that the analgesic effect of RXFP3 activation is specific to mechanical pain and does not affect thermal pain (**Figure 3.7B**). In contrast, mice injected with antagonist peptide and aCSF ($n = 7$ for saline, $n = 6$ for CFA) or agonist

(n = 5 for both groups) displayed decreased PWL following injection with either saline or CFA (Figure 3.7C-D).

Together, these data suggest that RXFP3 activity controls pain thresholds in a temporal manner, with pharmacological receptor activation alleviating mechanical pain, and inhibition of RXFP3 altering a potential effect of endogenous RLN3 signalling on normal pain thresholds.

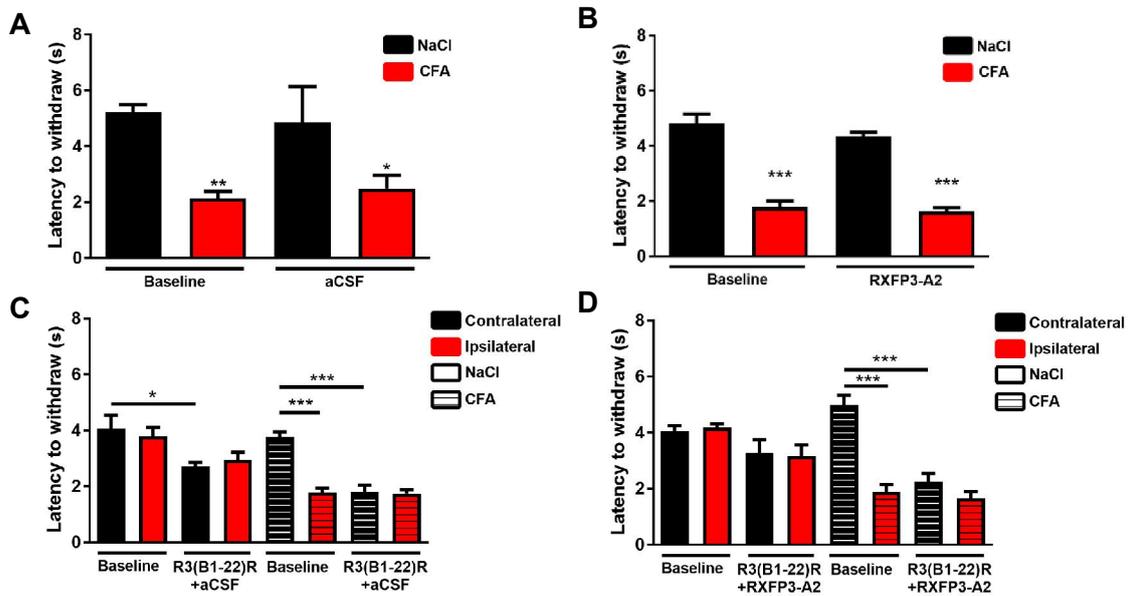


Figure 3.7 Effect of RXFP3 modulation on thermal pain thresholds. Mice were injected with saline (NaCl) in one hindpaw and CFA in the other (A, B) or injected with saline or CFA in one paw with no injection in the contralateral paw (C, D). For each cohort, a baseline test was performed 24 h before administering treatments and retesting. PWL is represented as “latency to withdraw” and expressed in s. Saline injection did not alter PWL, whereas injection of CFA decreased PWL consistent with thermal allodynia. Mice received the same treatments icv as in the von Frey test: (A) aCSF, (B) RXFP3-A2, (C) R3(B1-22)R + aCSF or (D) R3(B1-22)R + RXFP3-A2. Neither the injection of aCSF (A) or RXFP3-A2 (B) affected PWL. Injection of R3(B1-22)R and aCSF (C) induced a decrease in PWL in the contralateral paw (black columns) in mice injected with saline (full columns) or CFA (striped columns). Mice injected with CFA and treated with R3(B1-22)R and RXFP3-A2 (D) displayed decreased PWL in the contralateral paw. *p<0.05, **p<0.01, ***p<0.001 using two-way ANOVA followed by Bonferroni multiple comparison test for the effect of paw injection and treatment.

Another cohort of mice ($n = 5$ per group) was implanted with double-barrel cannulae targeting the ACC in both hemispheres. These mice were injected with CFA in the right hindpaw, which produced a significant decrease in PWT in the von Frey test (**Figure 3.8A**). Mice were then injected with aCSF or RXFP3-A2 into the ACC cerebral parenchyma. Neither treatment produced changes in PWT (**Figure 3.8A**), suggesting the previously observed analgesic effect is not due to direct activation of RXFP3 in the ACC.

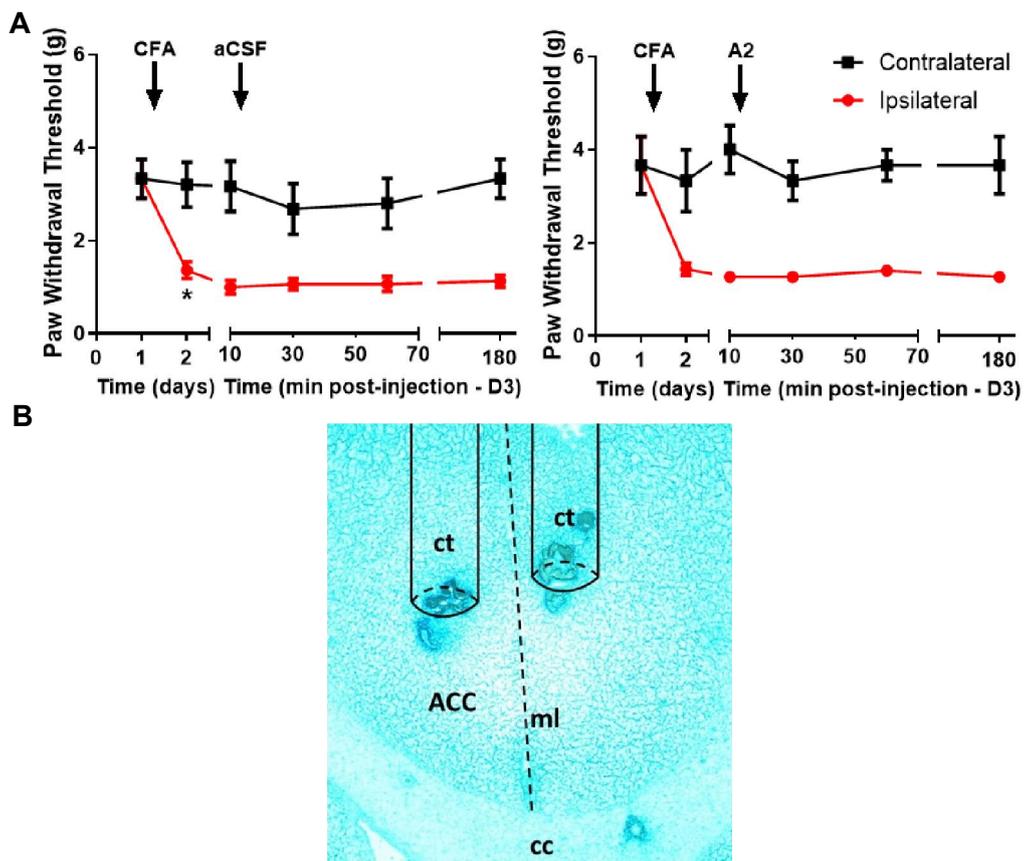


Figure 3.8. Activation of RXFP3 in ACC did not alter mechanical pain thresholds. Mice implanted with double-barrel guide cannulae targeting left and right anterior cingulate cortices were submitted to von Frey testing before and after the injection of i.pl. CFA. (A) CFA produced a significant decrease in PWT in the injected paw, whereas the intra-ACC injection of aCSF and RXFP3-A2 (A2 in the right graph) did not alter pain thresholds. Ipsilateral (red circles and lines) and contralateral paws (black squares and lines) were tested. (B) Example of a cannula targeting the ACC. cc: corpus callosum, ct: cannula tract; ml: midline.

3.3.2 Pain thresholds in RXFP3 KO mice

The potential role of tonic RXFP3 activity in pain modulation, was investigated using male and female RXFP3 KO mice. von Frey testing was performed before and after the injection of CFA (**Figure 3.9**). Two-way ANOVA analysis for the interaction between genotype and gender revealed no significant difference between WT males (n = 14 mice), KO males (n = 8 mice), WT females (n = 6 mice), or KO females (n = 11 mice). Moreover, both genotypes and sexes displayed decreased PWT after administration of CFA. These data suggest RXFP3 is not involved in tonic control of pain thresholds under normal conditions, or in control of nociceptive thresholds under inflammatory pain conditions (see Section 3.4 for further discussion).

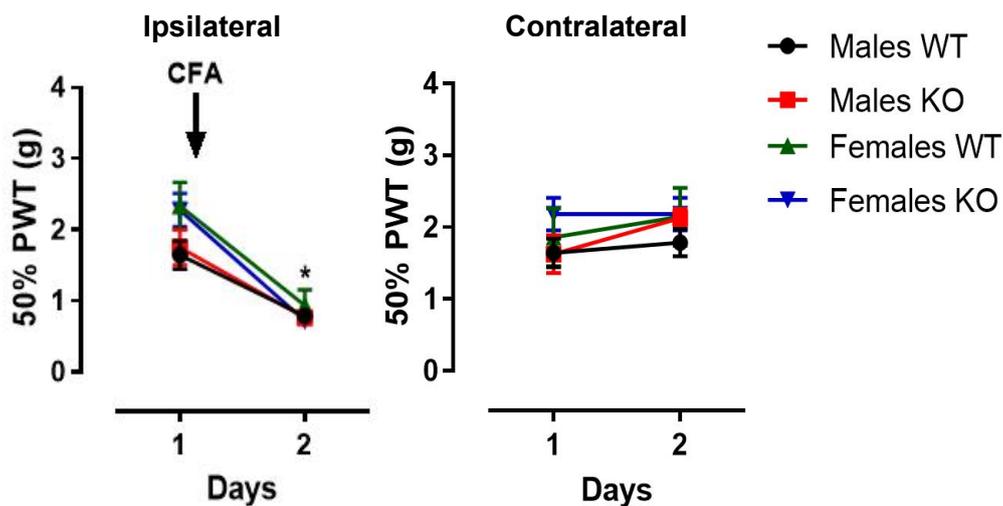


Figure 3.9. Male and female RXFP3KO mice display similar pain thresholds to those of WT mice.

Male and female WT and RXFP3KO mice were submitted to von Frey testing before and after the i.pl. injection of CFA in the right hindpaw. CFA produced a significant decrease in PWT as before, albeit no differences between the different groups were found. Graph legend is on the right side of the image.

* $p < 0.05$ using paired t-test for the effect of CFA.

3.3.2 Pharmacological studies in rats

In studies to investigate the role of RXFP3 modulation in persistent pain conditions, I implanted guide cannulae targeting the right LV in rats. These rats were submitted to von Frey testing once a week for 3 weeks after the injection of saline or CFA (**Figure 3.10**). After 3 weeks, aCSF (**Figure 3.10A**) vehicle or RXFP3-A2 (**Figure 3.10B**) were infused into the LV and PWT was measured 10, 30, 60, and 180 min after treatment. aCSF did not produce any changes in PWT in mice injected with saline (n = 5 rats) or CFA (n = 3 rats). Surprisingly, RXFP3-A2 did not alter PWT in saline- (n = 4 rats) or CFA-injected rats (n = 6 rats). This finding suggests that the mechanisms that allowed RXFP3 activation to elicit analgesia in rats 3-4 days after the injection of CFA (see Introduction to this Chapter) may not be sustained under persistent pain conditions (see also Section 3.4).

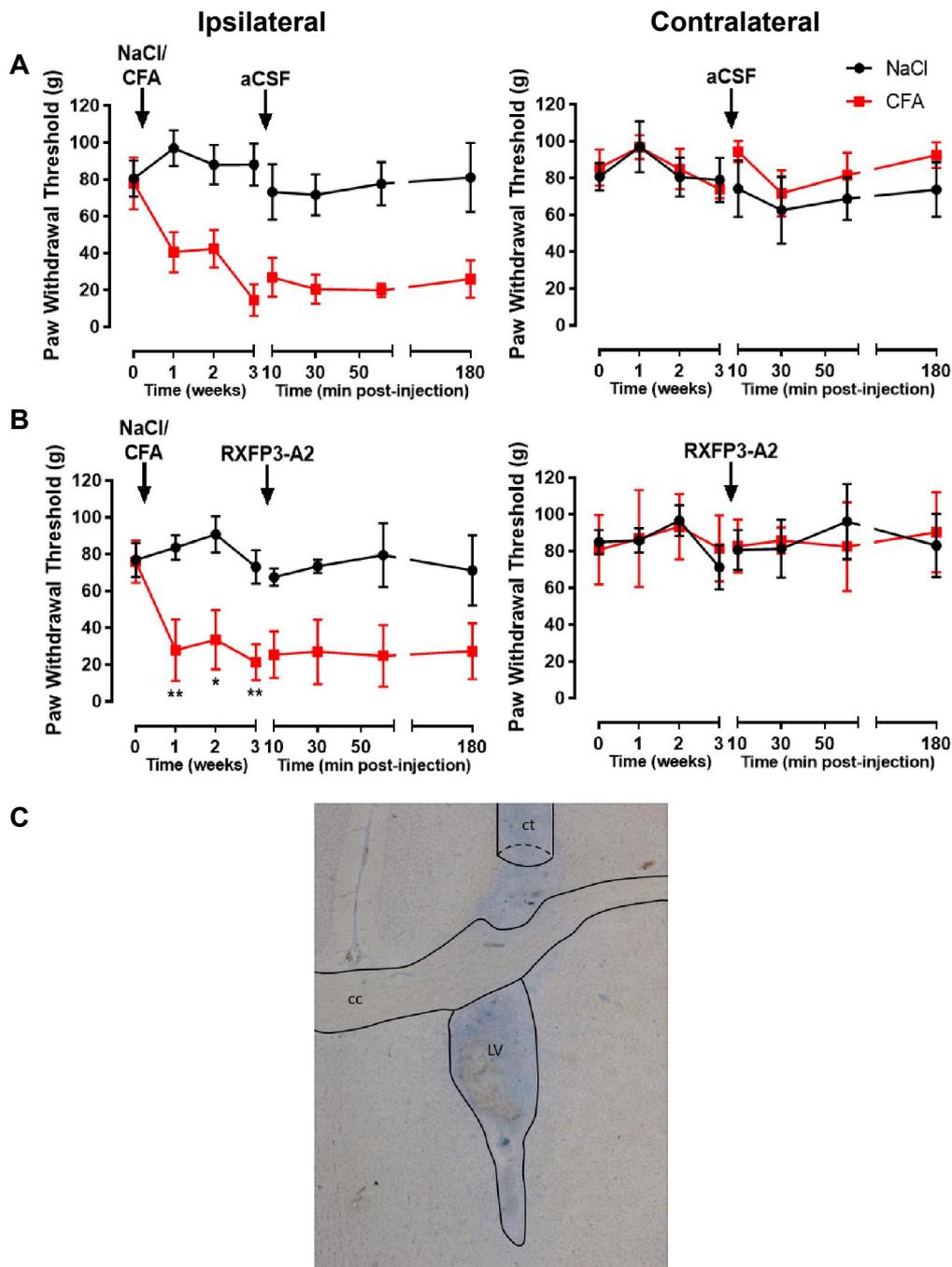


Figure 3.10. Activation of RXFP3 does not affect pain thresholds in rats 3 weeks post-CFA injection.

Rats (n = 3-6 per group) were implanted with cannulae targeting the right lateral cerebral ventricle and submitted to von Frey (A,B) testing before and after the i.pl. injection of saline (NaCl, black circles and lines) or CFA (red squares and lines). Rats were tested once a week for 3 weeks before treatments were administered icv. aCSF (A) or RXFP3-A2 (B) were administered through the guide cannula and tested for changes in PWT 10, 30, 60, and 180 min after injection. No changes were observed regardless of the treatment administered. (C) Example of correct cannula placement targeting the lateral cerebral ventricle. cc: corpus callosum; ct: cannula tract; LV: lateral ventricle.

3.4 Discussion

Persistent pain brings about changes in synaptic connections that contribute to its perpetuation, making its pathophysiology complex. In an effort to understand this complexity, key aspects of pain can be considered including the type of pain, its duration, and the presence of pre-existing conditions prior to the pain. Considering these variables can assist our understanding of how pain can be modulated and how manipulation of different neural signalling systems can affect pain thresholds. In this sense, preclinical models of pain allow researchers to freely manipulate these variables and determine the impact on nociception.

My research employed controlled manipulations of these variables with the purpose of furthering our understanding of how the RLN3/RXFP3 signalling system can influence nociception. In this context, I induced inflammatory pain (type of pain) in mice with no other pathology (no pre-existing conditions), and, using pharmacological approaches, tested the effects of modulating RXFP3 a short time after inducing pain (short duration). Using the von Frey test, I demonstrated that activation of RXFP3 produces mechanical analgesia, and that blocking the receptor generated behavioural hyperalgesia. Furthermore, I demonstrated that while activation of RXFP3 had no effect on thermal nociception, its blockade led to decreased PWL. These data indicate that while activation of RXFP3 produces mechanical analgesia, it does not elicit thermal analgesia, and suggests that endogenous RLN3 has a tonic effect on the control of both pain modalities. The apparent involvement of this signalling system in the control of one pain modality and not another suggests separate mechanisms are responsible for these types of pain. Indeed, our understanding of how distinct neural circuits regulate different pain modalities is still emerging. There is evidence that specific cell populations in the spinal cord encode how mechanical nociceptive information is transmitted to the brain (Peirs et

al., 2015; Seal et al., 2009). Indeed, compounds injected in the lateral ventricle can reach the spinal cord, at least at the cervical level, via the CSF (Proescholdt et al., 2000). However, RXFP3 is apparently not expressed by neurons in the spinal cord [(Liu et al., 2003b), see also ISH data from the Allen Brain Atlas (ABA) – www.brain-project.org], making it more likely that the observed effects involve modulation of descending pain pathways rather than local regulation of spinal circuits. In this regard, few studies have focused on elucidating the mechanisms by which different pain pathways influence distinct pain modalities. François and colleagues (2017) reported that a specialized subpopulation of GABA/ENK neurons in the RVM that project to the spinal cord specifically modulated mechanical pain, while not affecting thermal pain levels. Whether this neuron population is modulated by RXFP3 signalling directly or indirectly, via modulation of higher-order brain structures, remains to be elucidated. In this regard, the localized infusion protocol used to target the ACC provided some insight, by suggesting that the ACC is not strongly involved in the expression of the observed effects. However, like the RVM, the ACC may be a second-order structure that is influenced by RXFP3 activation/blockade. Regardless, the primary target region(s) for the analgesic effects of RXFP3 observed after icv administration of a selective agonist remain(s) to be identified. In a separate group of experiments, I tested the responses of RXFP3 KO mice to von Frey stimulation. These mice have a constitutive RXFP3 gene deletion, meaning that throughout their lifespan they will not express a functional receptor. In this context, I analysed differences between two genotypes and sexes (pre-existing conditions), but observed no changes in basal nociceptive thresholds or thresholds after injection of CFA. While this suggests that RXFP3 does not have an essential role in the control of nociceptive thresholds, we cannot exclude the possibility that compensatory mechanisms resulting from whole-of-life gene deletion may be involved (Dahlhoff et al., 2013;

Kawasaki et al., 2002), to maintain normal pain sensation even after exposure to CFA. In addition, under normal conditions or after exposure to restraint stress, these mice display similar degrees of saccharin and alcohol preference to WT mice (Walker et al., 2015). However, in the same study, Walker and colleagues demonstrated that when exposed to swim stress *and* restraint stress, RXFP3 KO mice had a decreased preference for alcohol compared to their WT littermates. These data provide evidence that behavioural differences between these genotypes may be masked, and perhaps can only be revealed by exposure to severe stressors, in line with earlier data related to the response of the RLN3/RXFP3 system to stress in rats (Philip J. Ryan et al., 2013; Tanaka et al., 2005) and mice (Zhang et al., 2015). Exposure to stressors such as restraint and swim stress protocols (Walker et al., 2015) may unmask phenotypic differences between the two genotypes. In this regard, pain-inducing protocols that elicit a higher degree of spontaneous pain may unmask such differences due to greater stress levels (Yeziarski and Hansson, 2018). These protocols include neuropathic pain models where spontaneous pain behaviours can be observed long after their establishment, as opposed to the CFA inflammatory pain model, where spontaneous pain behaviours are only observable in the day following injection (Djoughri et al., 2006).

Finally, I assessed the effect of RXFP3 activation on nociceptive responses in rats in a longer-lasting duration of pain protocol. In contrast to earlier findings in rats that were tested four days after CFA injection (K. Egron and S. Ma, see Section 3.1.2), administration of RXFP3-A2 did not alter pain thresholds in rats 3 weeks after injection of CFA. There are at least two possible explanations for these observations: (i) the availability of RXFP3 and/or its signalling machinery in the area(s) responsible the analgesic effect is altered, or (ii) the circuits responsible for this effect undergo neuroplastic changes, rendering RXFP3 signalling insufficient to produce analgesia.

Indeed, evidence exists that RXFP3 mRNA levels increase with age in different hippocampus and amygdala compartments (Meadows and Byrnes, 2015). However, we have no indication that these areas are responsible for the observed effects, and the fact that mRNA levels increase rather than decrease makes it more likely that RXFP3 signalling is not impaired. Therefore, the question remains whether RXFP3 expression levels and/or signalling are altered in the brain area(s) that lead to changes in pain thresholds in rats. On the other hand, as discussed, synaptic plasticity plays a critical role in the establishment and prolonging of pain in the brain and spinal cord (see Table 5.1 in Chapter 5). Whether similar mechanisms are at play and contribute to the loss of the analgesic effect of RXFP3 remains to be addressed. Lastly, the time window used to test how modulation of RXFP3 activity relates to nociception is a critical point in these experiments. While previous experiments in our laboratory were performed in a context of short-lasting pain, I assessed whether similar findings would be observed in a longer-lasting pain protocol. However, it was critical to choose a test time point that was beyond the acute pain window of s to days, but where recovery of pain thresholds due to latent sensitization had not yet been established. Latent sensitization is a chronic pain phenomena by which the injury elicits a period of hyperalgesia, followed by a recovery of normal pain thresholds, where hyperalgesia can be reinstated by exposure to stress (Chen et al., 2018; Marvizon et al., 2015). In the current study the aim was to test the effect of RXFP3 activation within the ‘hyperalgesia’ period that sets in after the injury, in this case the CFA injection. Nevertheless, it would be interesting to test whether RXFP3 can modulate pain behaviours after recovery of normal pain thresholds via latent sensitization. Centrally administering peptides before or after exposure to stress would allow assessment of whether RXFP3 can influence the effects of stress on pain, which if

positive, would be in line with the role the RLN3/RXFP3 system plays in stress responsiveness.

Together my findings indicate a role for the RLN3/RXFP3 system in the control of normal nociceptive thresholds. Additionally, I obtained evidence that modulation of RXFP3 is a viable target for therapeutic approaches directed at producing analgesia, at least in a short-lasting pain context.

Chapter 4 – Neuroanatomy and neurochemistry of RXFP3 networks in descending control brain areas

4.1 Introduction

A comprehensive understanding of a biological system requires extensive study of the separate components that comprise the system, and to determine how manipulations of a biological system affect an organism, distinct techniques are used to identify changes in behaviour, such as the approaches described in Chapters 3 and 5 - neuronal activity (e.g., electrophysiological and calcium imaging techniques), or macro/microstructure of neural circuits (e.g., volumetric analysis of brain regions and cytoarchitecture of nerve cells). One key component of such studies is the identification of the brain structures and cell types that mediate the expression of certain behaviours. At a microscopic level, single-cell phenotyping allows for a more detailed characterization of microcircuits and narrows the potential cellular targets, signalling partners, and possible behavioural outcomes. This Chapter comprises two separate studies that sought to (i) identify the brain regions mediating the anti-allodynic actions of RXFP3 activation, and (ii) characterize the neurochemical phenotype of Rxfp3 mRNA-containing neurons in these regions.

4.1.1 Distribution of RLN3/RXFP3 networks

Soon after the discovery of RLN3 (Bathgate et al., 2002), the major population of RLN3-expressing neurons was identified in the NI (Burazin et al., 2002). Later, smaller populations of RLN3-expressing neurons were described in the PAG, PnR, and an area dorsal to the *substantia nigra* (Tanaka et al., 2005). RLN3-immunoreactive nerve fibres were also detected in several regions of the rat and mouse brain, spanning from anterior compartments of the telencephalon, such as the olfactory bulb, to specialized nuclei of the midbrain and pons (Smith et al., 2010; Tanaka et al., 2005) (**Figure 4.1**).

Within the RLN3-expressing neuron populations, several studies have reported on specialized subpopulations that may have distinct functions. In the rat NI, all RLN3-expressing neurons have been reported to co-express the GABAergic neuron marker glutamate decarboxylase (GAD), with approximately 30% of these neurons co-expressing the Ca²⁺-binding protein calbindin (CB) D-28K (Ma et al., 2007). Tanaka and colleagues (Tanaka et al., 2005) reported that almost all RLN3-expressing neurons in the rat NI co-express type-1 receptors for the stress-responsive neuropeptide, CRF, and that exposure to swim stress increased expression of the activity-regulated transcription factor, c-Fos to a greater extent in double-immunopositive cells (i.e., RLN3 and CRF₁) than RLN3 only labelled neurons.

Diverse neuronal populations may differentially modulate NI neuron activity, as the NI receives input from orexin-expressing neurons of the lateral hypothalamus (Blasiak et al., 2015), as well as parvalbumin (PV)-, calretinin (CR)-, and CB-expressing neurons of the medial septum (MS) complex (Sánchez-Pérez et al., 2015). Conversely, RLN3 inputs from the NI impinge on CR- and CB-expressing neurons within the extended amygdala (Santos et al., 2016).

RXFP3 presents an equally widespread distribution pattern in rat (Ma et al., 2007) and mouse ((Smith et al., 2010), see also ISH data from the ABA – www.brain-map.org). In the rat MS complex, RXFP3 mRNA is expressed in GABAergic PV- and SST-expressing neurons, but not in choline acetyltransferase (ChAT)-positive neurons (Albert-Gascó et al., 2018).

The widespread distribution of RLN3/RXFP3 networks, paired with the distinct functions associated with the targeted regions, is in line with the findings that this system modulates several behavioural modalities (see section 3.1.2). Of interest to the study of pain, RLN3-containing fibres are present in rat brain areas such as the ACC, BNST,

paraventricular nucleus of the thalamus (PVT), PVN, CeA, and PAG (Ma et al., 2007; Tanaka et al., 2005), and RXFP3 mRNA expression is detected in these regions in both rat (Ma et al., 2007) and mouse ((Smith et al., 2010), see also ISH data from the ABA – www.brain-map.org). These data provide evidence that combining hodological and cytochemical data with pharmacological and functional results provides further insights into the mechanisms through which RLN3/RXFP3 networks modulate nociceptive thresholds.

4.1.2 Aims

The studies described in this Chapter aimed to (i) determine the brain areas involved in the analgesic effects of RXFP3 activation; and (ii) characterize the neurochemical phenotype of *Rxfp3* mRNA-containing neurons within these structures.

4.2 Methods

4.2.1 Immunohistochemistry (IHC)

Mice that had undergone behavioural testing following hindpaw injections of 0.9% NaCl or CFA, were centrally administered with the same treatments received initially (aCSF or RXFP3-A2) 1 h prior to being anaesthetized with lidocaine/pentobarbital, and transcardially-perfused with fresh 4% PFA. Brains were removed from these mice and post-fixed in 4% PFA at 4°C for 1.5 h. Brains were then placed in 30% sucrose in 0.1 M PBS solution at 4°C overnight; and then frozen on dry ice and kept at -80°C until use. Brains were then mounted in OCT (Tissue-Tek®, Sakura) and 20 µm sections were cut in the cryotome (Leica). Sections containing the ACC, anteromedial part of the BNST (amBNST), parataenial nucleus of the thalamus (PT), and lateral, basolateral, and central amygdala (LA, BLA, and CeA, respectively) were submerged in 0.1 M PBS and later preserved in cryoprotection solution consisting of 10% glycerol (Sigma-Aldrich) and 25% sucrose (Sigma-Aldrich) in 0.1 M PBS and frozen at -80°C.

For IHC experiments, sections were thawed and washed in 0.1 M PBS for 3 × 5 min. Sections were incubated with blocking solution consisting of 1% goat normal serum and 0.25% Triton X-100 (Sigma-Aldrich) in 0.1 M PBS for 1 h. The IHC reaction was initiated by the addition of rabbit anti-c-Fos primary antibody (1:500, Santa-Cruz, SC-52) to the incubation. After overnight incubation, the reaction was stopped by washing the sections in 0.1 M PBS for 3 × 5 min. The secondary incubation was conducted in 0.1 M PBS containing 1% goat normal serum, 0.25% Triton X-100 (Sigma-Aldrich), and Alexa 568-conjugated goat anti-rabbit secondary antibody (1:500; Molecular Probes, Eugene, OR, A-11011). After washing in 0.1 M PBS, sections were mounted on SuperFrost slides with Fluoromount-G/DAPI (ThermoFisher Scientific) and air dried overnight in the dark. Slides were kept at -20°C until examined.

4.2.2 *In situ* hybridization (ISH)

ISH was performed using the RNAscope® Fluorescent Multiplex Assay from Advanced Cell Diagnostics (ACD, Newark, CA, USA) as per manufacturer's instructions, with minor modifications. Naïve mice (n = 3) were anaesthetized with 4% isoflurane, decapitated, and their brains removed. Brains were rinsed in PBS before being frozen on dry ice. The brains were then mounted in OCT embedding gel (Tissue-Tek®, Sakura) and sectioned in a cryostat (Leica). Sections containing the ACC, BNST, thalamus, amygdala, and PAG were mounted on SuperFrost Plus slides (ThermoFisher Scientific) and submerged in 4% PFA for 16 min. After washing in RNase-free PBS for 1 min, the sections were dehydrated by incubation in increasing concentrations of ethanol (50, 70, 100%) for 5 min each. Slides were stored in 100% ethanol at -20°C overnight. The next day, slides were air dried for 10 min, then a waterproof barrier was drawn around the sections using a hydrophobic wax pen (ImmEdge Hydrophobic Barrier Pen, Cat #310018), which was air-dried for 10 min. Sections were incubated with Protease Pretreat-4 (Cat#322340) at room temperature for 20 min and then washed using wash buffer solution (Cat#310091) diluted 1:20 in 0.1 M PBS. Sections were incubated with a combination of three probes targeting *Rxfp3* (Cat#439381), *Sst* (Cat#404631-C2), and *Pvalb* (Cat#421931-C3) mRNA for 2 h at 40°C. Signals were amplified using ACD amplifying reagents following the manufacturer's protocol. Finally, slides were incubated with DAPI (#320851), mounted with Fluoromount (ThermoFisher Scientific), and air-dried overnight in a cassette to avoid light exposure. Slides were stored at -20°C until use.

4.2.3 Image acquisition

Slides were examined under a confocal microscope (Leica TCS SPE, Mannheim, Germany). For IHC slides, DAPI and Alexa 568 were excited using 430 nm and 561 nm lasers, respectively. For ISH slides, DAPI, Alexa 488, ATTO 550, and ATTO 647 were excited using 430 nm, 488 nm, 561 nm and 635 nm lasers, respectively. For quantitative analysis, 8-35 single optical sections were acquired in z-stacks with 500 nm steps with a 40× (IHC) or 63× (ISH) objective. Tile scans were acquired by collecting 6 optical sections in z-stacks in a series of 21-68 adjacent planes, using a 20× objective and a zoom factor of 1.5. Gain (800-1000V) and offset (-0.9 to -1.8%) were adjusted for each acquisition.

4.2.4 Image analysis

Analysis of co-localization and cell quantification in ISH experiments was performed manually on digitized images using ImageJ software. A cell represented by a DAPI-stained nucleus was considered positive when it contained at least 2 ‘grains’ of a given mRNA species. *Rxfp3* and *Sst* mRNA-related ‘grains’ that did not co-localize with DAPI were counted towards the total number of grains of their respective species for the closest nucleus; whereas *Pvalb* mRNA ‘grains’ that did not co-localize with DAPI were not considered, as this mRNA species is commonly found outside the soma of neurons.

For IHC experiments, quantification of the total number of DAPI-stained nuclei was performed using the 3D Tissue Spatial Analysis tool of ImageJ software. This method yielded an approximate 2% error rate when compared to manual counting, measured in four different fields of view for each of eight images, by two independent observers. The total number of Fos-immunoreactive nuclei (‘number of objects’) and intensity of staining in each nucleus (‘intensity of objects’) were determined using a custom-written macro.

Briefly, the macro program eliminated bright spots that were too small to correspond to nuclei, smoothed the edges of each object, and converted them to binary (black and white) to generate a map for the location of each object. This map was then used to count the number of objects, and superposed to the original image, to determine the corresponding intensity of the counted objects. This method yielded an approximate error rate of 5% when compared to manual counting, measured in four different fields of view for each of eight images, by two independent observers.

4.2.5 Statistical analysis

Statistical analysis of IHC data was performed using R software (www.r-project.org). A coefficient of variation was used as a measure of data dispersion to determine the dispersion of staining intensity. To achieve this, the intensity of each object within an image was normalized to the minimum intensity within that image. Comparisons of the mean percentage of Fos-immunoreactive cell nuclei and coefficient of variation of staining intensity were performed using non-parametric Wilcoxon test to test for differences between groups (effect of ‘treatment’). When testing for differences between hemispheres (effect of ‘side’), the Wilcoxon signed-rank test for paired samples was used; and as no differences were found, the data were pooled for graphical representation. Significance level was fixed at $p < 0.05$.

4.3 Results

4.3.1 Fos IHC studies

In these studies, the surrogate neuronal activity marker, Fos-immunoreactivity (Fos-IR) was used to assess the effect of subcutaneous CFA injection and/or icv RXFP3-A2 injection on the activity within six different brain regions: ACC, amBNST, PT, LA, BLA, and CeA. These areas were chosen for their involvement in pain transmission and abundance of RLN3 fibres and/or Rxfp3 mRNA (see Introduction in this Chapter). The total number of Fos-IR-stained nuclei and the average intensity of Fos-IR in each neuronal nucleus were quantified and compared as mean values of total number of objects and a coefficient of variation of staining intensity, respectively, for each area. This analysis revealed no significant differences in the number of Fos-immunoreactive cells (**Table 4.1**, see Appendix 1 for complete statistical analysis results) or the average intensity of Fos-IR (**Table 4.2**) between the different experimental conditions.

Table 4.1. Effect of RXFP3 activation on c-Fos expression in different brain regions.

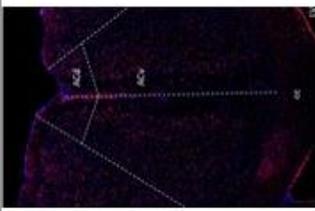
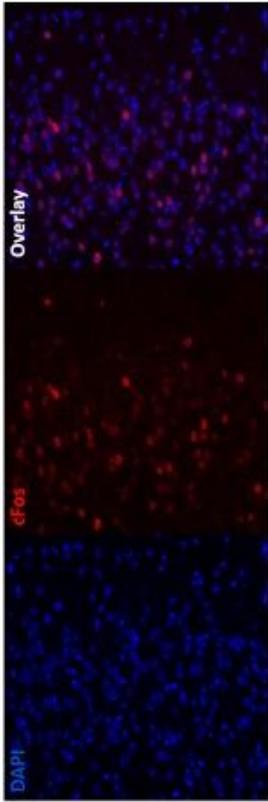
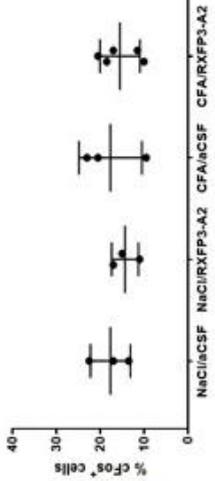
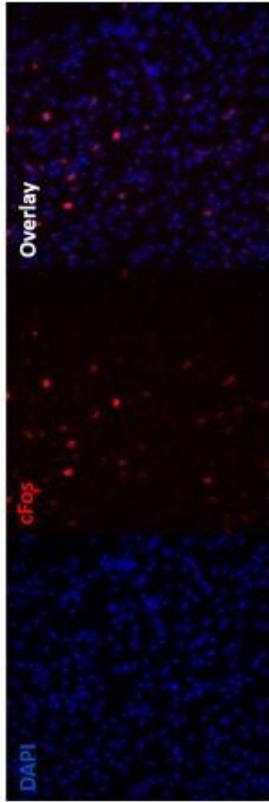
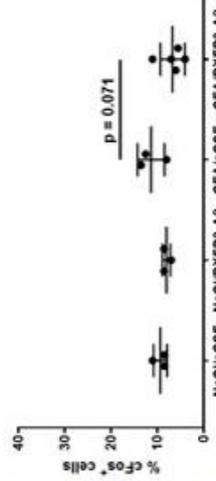
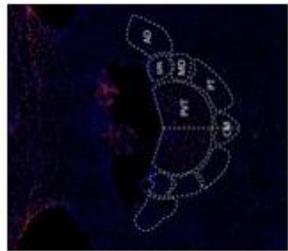
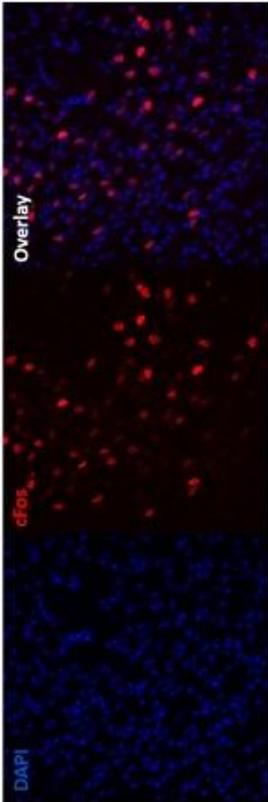
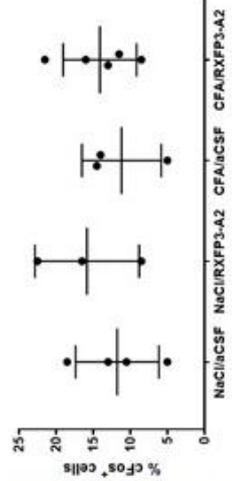
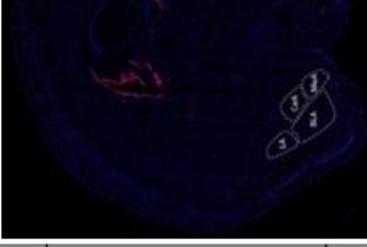
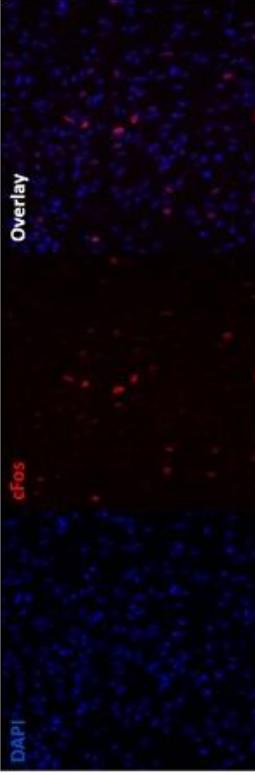
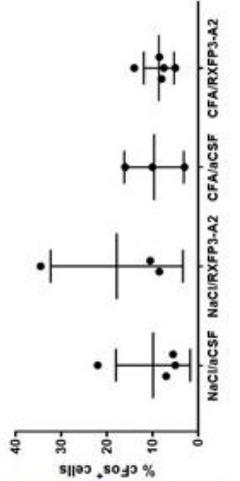
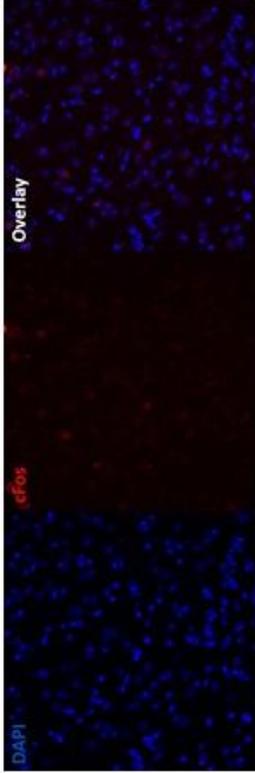
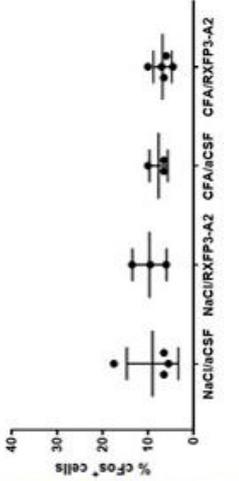
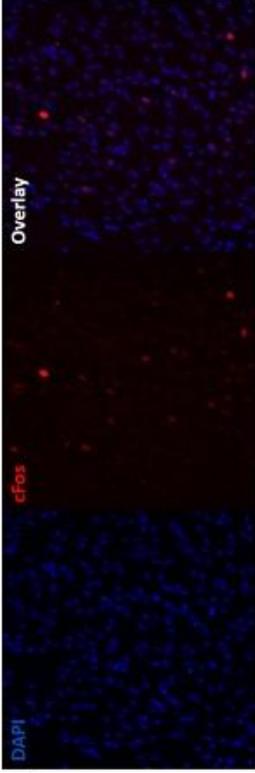
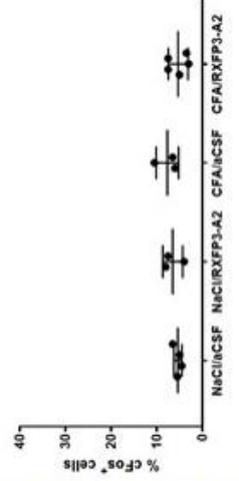
Brain area	Overview	c-Fos-IR	Fos-IR cell quantification
ACC			
amBNST			
PT			

Table 4.1. (continued)			
Brain area	Overview	cFos-IR	Fos-IR cell quantification
LA			
BLA			
CeA			

Abbreviations: ACC – anterior cingulate cortex; ACd – dorsal part of the ACC; aco – anterior commissure; ACv – ventral part of the ACC; AD – anterodorsal nucleus of the thalamus; alBNST – anterolateral part of the bed nucleus of the stria terminalis; amBNST – anteromedial part of the BNST; BLA – basolateral amygdala; cc – corpus callosum; CeA – central amygdala; CM – centromedial nucleus of the thalamus; fx – fornix; LA – lateral amygdala; LV – lateral ventricle; LSr – rostral part of the lateral septum; LSV – ventral part of the LS; MD – mediodorsal nucleus of the thalamus; MeA – medial amygdala; ovBNST – oval part of the BNST; PT – parataenial nucleus of the thalamus; PVT – paraventricular nucleus of the thalamus.

Table 4.2. Comparison of coefficient of variation of mean density of Fos-immunoreactive cells by brain area

Brain area	Condition	n	SR	C	CR	p-value
ACC	NaCl/aCSF	3	9	6	[0] U [9]	0.700
	NaCl/RXFP3-A2	3	12			
amBNST	NaCl/aCSF	3	10	5	[0] U [9]	0.990
	NaCl/RXFP3-A2	3	11			
PT	NaCl/aCSF	4	14	8	[0-1[U]11-12]	0.629
	NaCl/RXFP3-A2	3	14			
LA	NaCl/aCSF	4	17	5	[0-1[U]11-12]	0.857
	NaCl/RXFP3-A2	3	11			
BLA	NaCl/aCSF	4	17	5	[0-1[U]11-12]	0.85
	NaCl/RXFP3-A2	3	11			
CeA	NaCl/aCSF	4	18	4	[0-1[U]11-12]	0.629
	NaCl/RXFP3-A2	3	10			
ACC	CFA/aCSF	3	17	11	[0-1[U]14-15]	0.393
	CFA/RXFP3-A2	5	19			
amBNST	CFA/aCSF	3	16	10	[0-1[U]14-15]	0.571
	CFA/RXFP3-A2	5	20			
PT	CFA/aCSF	3	9	3	[0-1[U]14-15]	0.250
	CFA/RXFP3-A2	5	27			
LA	CFA/aCSF	3	12	6	[0-1[U]14-15]	0.786
	CFA/RXFP3-A2	5	24			
BLA	CFA/aCSF	3	14	8	[0-1[U]14-15]	0.990
	CFA/RXFP3-A2	5	22			
CeA	CFA/aCSF	3	12	6	[0-1[U]14-15]	0.786
	CFA/RXFP3-A2	5	24			
ACC	NaCl/aCSF	3	10	5	[0] U [9]	0.990
	CFA/aCSF	3	11			
amBNST	NaCl/aCSF	3	13	7	[0] U [9]	0.400
	CFA/aCSF	3	8			
PT	NaCl/aCSF	4	16	6	[0-1[U]11-12]	0.990
	CFA/aCSF	3	12			
LA	NaCl/aCSF	4	19	3	[0-1[U]11-12]	0.400
	CFA/aCSF	3	9			
BLA	NaCl/aCSF	4	17	5	[0-1[U]11-12]	0.857
	CFA/aCSF	3	11			
CeA	NaCl/aCSF	4	18	4	[0-1[U]11-12]	0.629
	CFA/aCSF	3	10			

(continued next page)

Table 4.2 (continued)

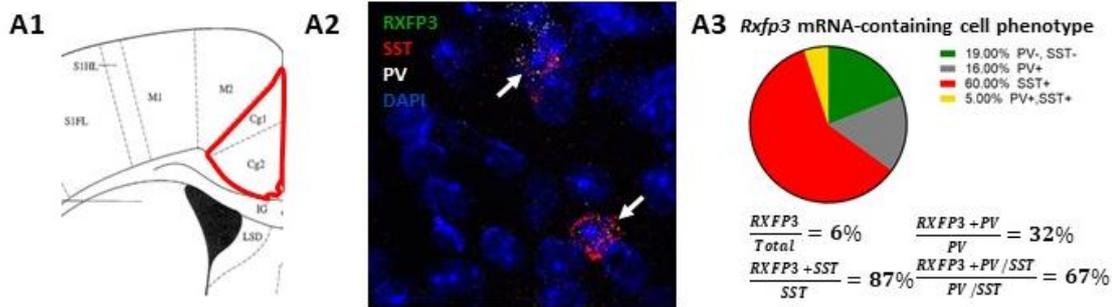
ACC	NaCl/RXFP3-A2	3	19	13	[0-1[U]14-15]	0.143
	CFA/RXFP3-A2	5	17			
amBNST	NaCl/RXFP3-A2	3	19	13	[0-1[U]14-15]	0.143
	CFA/RXFP3-A2	5	17			
PT	NaCl/RXFP3-A2	3	16	10	[0-1[U]14-15]	0.571
	CFA/RXFP3-A2	5	20			
LA	NaCl/RXFP3-A2	3	14	8	[0-1[U]14-15]	0.990
	CFA/RXFP3-A2	5	22			
BLA	NaCl/RXFP3-A2	3	15	9	[0-1[U]14-15]	0.786
	CFA/RXFP3-A2	5	21			
CeA	NaCl/RXFP3-A2	3	13	7	[0-1[U]14-15]	0.990
	CFA/RXFP3-A2	5	23			

C – test result; CR – critical region; n – number of mice; SR – sum of ranks

4.3.2 ISH studies

In these studies, ISH was used to identify the neuronal populations that express *Rxfp3* mRNA in nine different brain areas: ACC, amBNST, PT, anterodorsal nucleus of the thalamus (ADT), PVT, LA, BLA, CeA, and vIPAG. In the ACC (**Figure 4.2A**), the majority of *Rxfp3* mRNA-expressing neurons co-expressed *Sst* (60% of total neurons) and represented ~87% of the SST interneuron population. In the BNST (**Figure 4.2B**), *Sst* mRNA was detected in 27% of *Rxfp3* mRNA-expressing neurons, and the latter was detected in 75% of *Sst* mRNA-expressing neurons. These data suggest that RXFP3 is expressed by most SST-expressing neurons in this region, and that the neurochemical phenotype of the majority of *Rxfp3* mRNA-expressing neurons remains to be elucidated. The thalamus is an important relay for ascending and descending pain pathways, with several nuclei involved in sensory perception of pain (Yen and Lu, 2013). In these studies, three nuclei that contain a high density of *Rxfp3* mRNA were examined. In the PVT (**Figure 4.3A**), most (~69%) *Rxfp3* mRNA-expressing neurons co-expressed *Pvalb* mRNA. In contrast, no *Sst* mRNA-positive neurons were detected, in line with the reported distribution for this mRNA species (ABA ISH data – www.brain-project.org). In the ADT (**Figure 4.3B**), 85% of *Rxfp3* mRNA-expressing neurons co-expressed *Pvalb* mRNA. Notably, *Rxfp3* mRNA-related fluorescence was mainly observed in cells in the inferomedial portion, in the vicinity of the *stria medularis*, where they exclusively co-expressed *Pvalb* mRNA. Despite this, the quantification of *Rxfp3* mRNA-expressing neurons (**Figure 4.3B3**) describes the total number of cells in the ADT, and not just this small sub-population of neurons. In the PT (**Figure 4.3C**), 72% of *Rxfp3* mRNA-expressing neurons co-expressed *Pvalb* mRNA.

A – ACC



B – amBNST

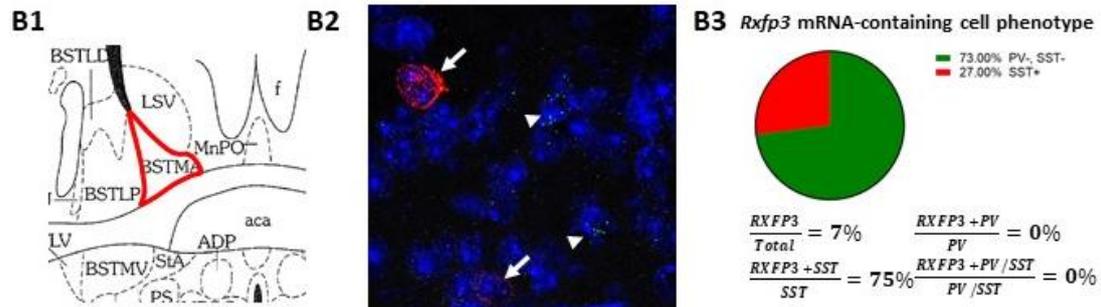
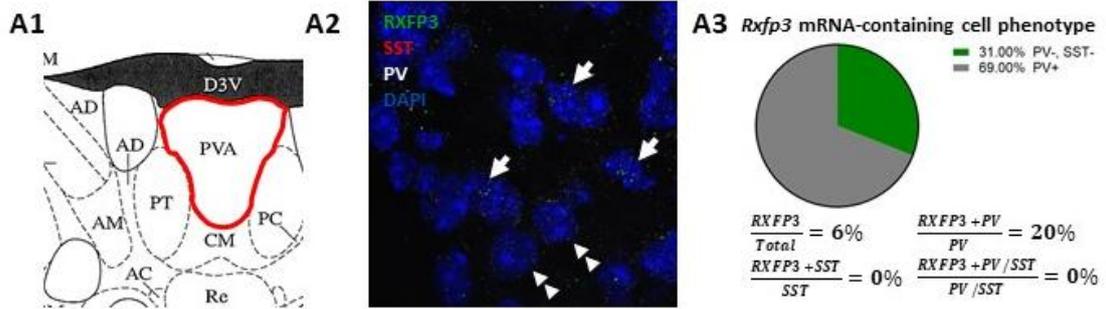


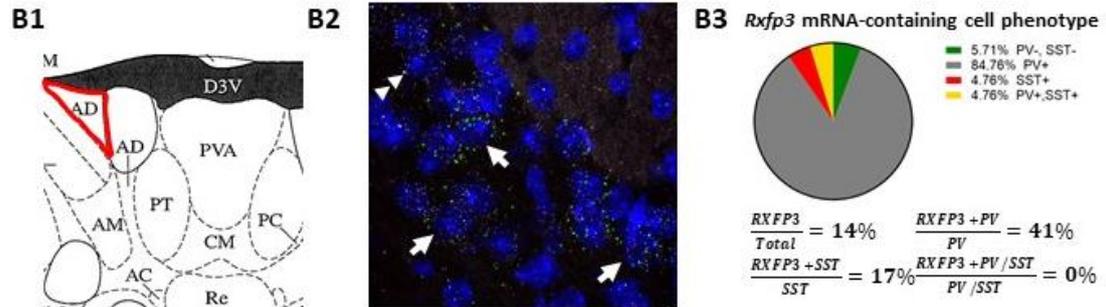
Figure 4.2. Neurochemical profile of *Rxfp3* mRNA-containing neurons in the ACC and amBNST.

ISH studies were performed to characterize the phenotype of *Rxfp3* mRNA-containing neurons in (A) the ACC and (B) amBNST. (A1, B1) Schematic representations of the areas examined (highlighted in red) were adapted from the mouse brain stereotaxic coordinates atlas (Paxinos and Franklin (2004)). (A2, B2) Representative photomicrographs for each area contain cell nuclei (DAPI, blue) where *Rxfp3* mRNA (green) co-localizes with *Sst* mRNA (red) (arrows) or with neither of the marker transcripts used (arrow heads). None of the neurons examined co-expressed *Rxfp3* and *Pvalb* mRNA (white). (A3, B3; top) A pie chart of the relative co-expression of *Pvalb* and *Sst* mRNA in *Rxfp3* mRNA-containing neurons. (A3, B3; bottom) Proportion of *Rxfp3* mRNA-containing neurons in relation to total cells in the area of interest, and proportion of *Rxfp3* mRNA co-labelled cells in relation to the total number of neurons containing the respective mRNA species.

A – PVT



B – ADT



C – PT

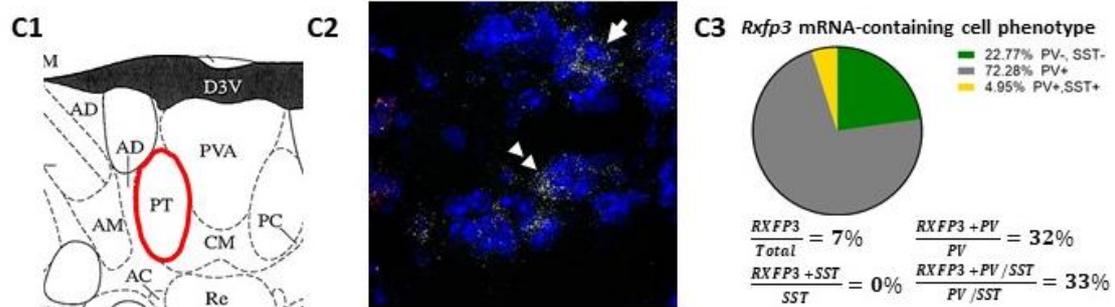


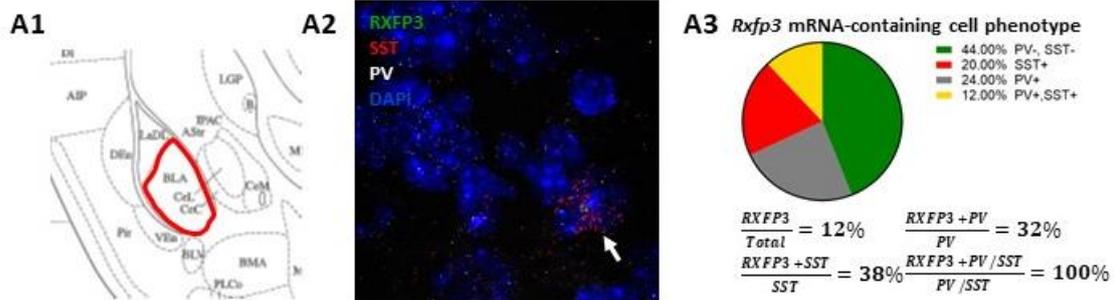
Figure 4.3. Neurochemical profile of Rxfp3 mRNA-containing neurons in the PVT, AD and PT. ISH studies were performed to characterize the phenotype of *Rxfp3* mRNA-containing neurons in the (A) PVT, (B) ADT and (C) PT. (A1, B1, C1) Schematic representations of the areas examined (enclosed by red lines) were adapted from the mouse brain stereotaxic coordinates atlas by Paxinos and Franklin (2004). (A2, B2, C2) Representative photomicrographs from each area contain cell nuclei (DAPI, blue) where *Rxfp3* mRNA (green) co-localizes with *Pvalb* mRNA (white) (large arrows), or where *Pvalb* was detected alone (double arrowheads). None of the nuclei represented co-expressed *Rxfp3* and *Sst* mRNA (red). (A3, B3, C3; top) A pie chart of the relative co-expression of *Pvalb* and *Sst* mRNA in *Rxfp3* mRNA-containing neurons. (A3, B3, C3; bottom) Proportion of *Rxfp3* mRNA-containing neurons in relation to total cells in the area of interest, and proportion of *Rxfp3* mRNA co-labelled cells in relation to the total number of neurons containing the respective mRNA species.

The amygdala contains distinct, specialized nuclei involved in nociception, both in its sensory-discriminative and aversive components (Veinante et al., 2013), each of them with different RXFP3 expression profiles in the mouse (Smith et al., 2010). In the material examined, the LA lacked *Rxfp3* mRNA (data not shown), and so no quantification was performed for this region. However, the BLA and CeA (**Figure 4.4A and B**) contained distinct profiles of *Rxfp3* mRNA-expressing neurons, with neurons co-expressing *Pvalb* or *Sst* mRNA, or both marker transcripts, to varying degrees. In both regions, a considerable proportion of neurons did not express either *Pvalb* or *Sst* mRNA (44% for BLA, 35% for CeA). Thus, the chemical phenotype of these cells remains to be elucidated.

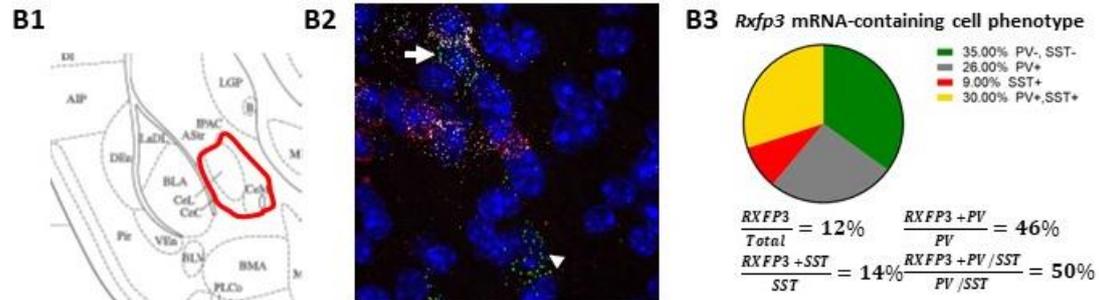
Similarly, in the vIPAG, one of the key relays for descending pain pathways, *Rxfp3* mRNA was co-expressed with *Pvalb* (31%) and *Sst* (10%) mRNA alone, or in combination (21%), with a remaining significant proportion of neurons (38%) lacking these markers (**Figure 4.4C**).

Collectively, these data are consistent with RXFP3 signalling being able to modulate distinct neuronal populations in multiple brain regions, including SST-expressing neurons in the ACC and PV-expressing neurons in the thalamus. At present, the precise neurochemical profile of the majority of *Rxfp3* mRNA-expressing neurons in other regions, such as the extended amygdala and vIPAG, are yet to be characterized. Further efforts to elucidate these details in additional multiplex ISH studies will provide insights into other potential cellular targets for RXFP3 modulation.

A – BLA



B – CeA



C – vIPAG

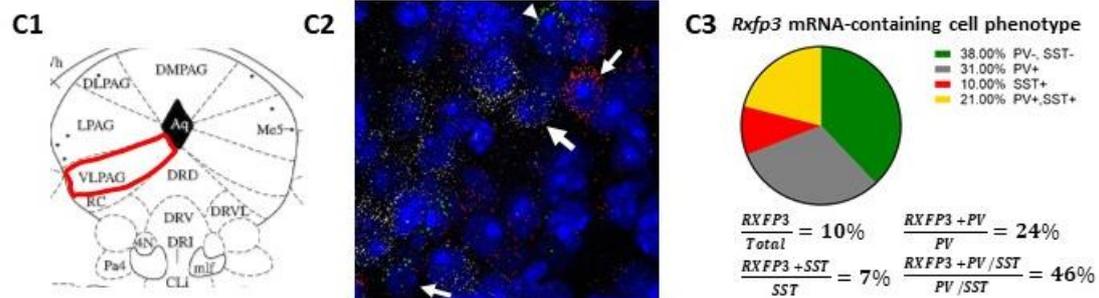


Figure 4.4. Neurochemical profile of *Rxfp3* mRNA-containing neurons in the BLA, CeA and vIPAG.

ISH studies were performed to characterize the phenotype of *Rxfp3* mRNA-containing neurons in the (A) BLA, (B) CeA, and (C) vIPAG. (A1, B1, C1) Schematic representations of the areas examined (highlighted in red) were adapted from the mouse brain stereotaxic coordinates atlas by Paxinos and Franklin (2004). (A2, B2, C2) Representative photomicrographs for each area contain cell nuclei (DAPI, blue) where *Rxfp3* mRNA (green) co-localizes with *Sst* mRNA (red) (arrows), *Pvalb* mRNA (white) (large arrows), or neither of the markers (arrowheads). (A3, B3, C3; top) A pie chart of the relative co-expression of *Pvalb* and *Sst* in *Rxfp3* mRNA-containing neurons. (A3, B3, C3; bottom) The proportion of *Rxfp3* mRNA-containing neurons in relation to total cells in the area of interest, and proportion of *Rxfp3* mRNA co-labelled cells in relation to the total number of neurons containing the respective mRNA species.

4.4 Discussion

The aim of the initial neurochemical studies described in this Chapter was to identify brain regions involved in the pharmacological effects of RXFP3 modulation to produce behavioural analgesia/hyperalgesia (see Chapter 3). However, the immunohistochemical detection of Fos protein in putative target areas was unable to identify a region or regions where the number of Fos-IR⁺ nuclei or Fos-IR staining intensity were altered by the pharmacological manipulations (**Table 4.1**). Therefore, it is important to consider some of the likely reasons why these experiments did not clarify the issue.

Firstly, the areas examined are composed by different neuronal populations, which may have distinct roles in nociception. For example, the ACC comprises excitatory (glutamatergic) principal cells, as well as multiple types of inhibitory, GABAergic neurons (e.g. PV-, SST- and 5-hydroxytryptamine 3a receptor (5-HT_{3a}R)-positive GABAergic interneurons (Rudy et al., 2011)). Given that the putative target neurons for RXFP3 modulation in this area are mainly PV- and SST-expressing interneurons (**Figure 4.2**), it is possible that alterations in Fos-IR in the current study are masked by the consideration of all neuron populations, regardless of neurochemical phenotype. Further studies that include double- or triple-labelling of neurons in the regions examined, using PV and SST, in addition to, Fos-IR, might help clarify this issue.

Secondly, a large number of brain regions are involved in descending pain modulation, and the present study did not consider all potential targets. Other areas of the cortex (e.g. somatosensory and insular cortices), compartments of the BNST, namely the alBNST (Ide et al., 2013; Kaneko et al., 2016; Tran et al., 2014), as well as thalamic sub-nuclei (see below) are known to modulate nociceptive responses. Whether one or more of these areas would display significant changes in activity as reflected by Fos-IR after RXFP3-A2 treatment remains to be explored.

Another key factor to consider is the time course used in the experiments. As Fos is a transiently-expressed protein (Nahm and Noebels, 1998), the time point at which it reaches peak expression is important for such activity-tracking studies. For this study, we chose to assess Fos-IR 1 h after treatment, as it was in line with the time window of 20-90 min previously used to study the effects of RXFP3-A2 administration on behaviour and expression of another neuronal activity marker, pERK (Albert-Gascó et al., 2017). However, these markers may reach peak expression over different timescales in response to receptor stimulation, which may explain why the chosen time point did not identify any differences in Fos-IR. Future studies could assess Fos-IR in response to RXFP-A2 treatment over a wider time frame, or use alternate markers, such as pERK or other transcription factor genes (Albert-Gascó et al., 2017).

Lastly, a key weakness of this study was that the sample size was probably too small to provide sufficient statistical power, and this possibility could best be tested in future studies of a larger number of mice, in addition to the improved design described above. A plethora of brain areas and neural circuits contribute to the processing of nociceptive signals, with different neuronal populations playing distinct roles in decoding noxious stimuli. These neuron populations can be distinguished by the neurochemical profile, either by expression of a specific protein or mRNA species. As such, the current studies aimed to characterize the neurochemical profile of *Rxfp3* mRNA-containing neurons, by examining the expression profile of *Sst* and *Pvalb* mRNA in these neurons in nine brain areas known to influence pain thresholds.

In the cerebral cortex, PV- and SST-expressing neurons account for a large portion of the GABAergic interneuron population, reportedly comprising ~70% of neocortical interneurons (Rudy et al., 2011). *Rxfp3* mRNA has previously been detected in GABAergic interneurons of the hippocampus (Rytova et al., 2019) or rats and MS

complex of rats (Albert-Gascó et al., 2018) and mice (Haidar et al., 2019). Thus, *Pvalb* and *Sst* mRNA are ideal candidates for likely co-expression with *Rxfp3* mRNA in the cerebral cortex. Indeed, in the ACC, neurons expressing one or other of these transcripts comprised ~81% of the *Rxfp3* mRNA-containing cells, with most neurons (~60%) co-expressing *Sst* mRNA. In pharmacological studies in mice (Section 3.3.1), there was no effect of RXFP3 activation in the ACC on mechanical pain thresholds, and the present data suggest that most neurons targeted by icv injection of RXFP3-A2 are SST-positive interneurons in ACC and elsewhere. To the best of my knowledge, no studies have reported on the effect of inhibition of these interneuron populations on pain, which is the likely outcome of RXFP3 activation given that the receptor couples to G_{i/o}-proteins, with negative coupling to AC (Liu et al., 2003b) and RXFP3-A2 has been shown to inhibit hypothalamic neurons (Kania et al., 2017). However, optogenetic activation of SST-expressing neurons of the ACC did not alleviate CFA-induced allodynia, while the same activation protocol elicited analgesia when targeting PV-expressing interneurons (Kang et al., 2015). Thus, it is possible that localized or broad inhibition of SST-expressing interneurons has no detectable effect on pain thresholds, although further studies are required to clarify this issue.

In the amBNST, *Sst* mRNA was present in ~27% of *Rxfp3* mRNA-containing neurons. A recent study reported that *vGluT2* mRNA-containing neurons account for ~35% of *Rxfp3* mRNA-containing cells in the mouse BNST (Ch'ng et al., 2019). The same study observed that the degree of co-expression of these two mRNA species varied in a rostral-to-caudal gradient (Ch'ng et al., 2019). It is unlikely that these inhibitory and excitatory neuron populations overlap given that the amBNST is mostly composed of GABAergic neurons that express ENK or dynorphin (Poulin et al., 2009). Moreover, *Pvalb* mRNA was not found in the amBNST, which is in line with ISH data from the ABA (www.brain-

[map.org](#)). This suggests that other markers, such as GABA-related enzymes, ENK, and dynorphin may be suitable targets for the further study of *Rxfp3* mRNA co-expression. Whether the degree of co-expression of *Sst* and *Rxfp3* mRNA varies in rostral and caudal parts of the amBNST remains to be studied.

The thalamus comprises several specialized nuclei that process both ascending and descending nociceptive information (Yen and Lu, 2013). Previous studies have observed that the mediodorsal (MD), centromedial (CM), and PVT nuclei are enriched in *Rxfp3* mRNA in the mouse (Smith et al., 2010), and these structures play a role in descending pain modulation (Yen and Lu, 2013). In these studies, the co-expression of *Rxfp3* mRNA with *Sst* and *Pvalb* mRNA was examined. Most (~70-85%) *Rxfp3* mRNA-containing neurons co-expressed *Pvalb* mRNA, suggesting a high, albeit varying, degree of co-expression of the two markers. To the best of my knowledge, there are no studies of the role of these thalamic PV-expressing neuron populations in pain transmission. Further studies are warranted to elucidate the role of RXFP3 modulation in these thalamic regions, including any effects on descending pain modulation.

The amygdala comprises different nuclei, all of which influence nociceptive transmission and ascending and descending pain modulation. Specifically, numerous studies have found that modulation of neuropeptide signalling in the BLA and CeA influence pain thresholds (see Section 2.2). However, the role of the RLN3/RXFP3 system has not been previously investigated. My findings suggest that RXFP3 is expressed by distinct neuronal populations in both these regions, where *Rxfp3* mRNA is co-expressed with *Sst* and *Pvalb* mRNA. Although their role in nociception has yet to be investigated, PV-expressing GABAergic interneurons of the BLA displayed decreased firing activity during the presentation of a painful conditioned stimulus (footshock) (Wolff et al., 2014), thus likely gating associative learning to aversive stimuli.

Conversely, SST-expressing GABAergic neurons of the CeA are inhibited upon presentation of an innocuous conditioning stimulus (auditory cue) during fear recall, suggesting synaptic plasticity changes during conditioning occur during associative learning (Yu et al., 2016). These phenomena relate to fear conditioning, which has been suggested to be strongly linked to nociception and nocifensive behaviours ((Butler and Finn, 2009), see also sections 2.2 and 5.1.2). Whether these findings relate to potential changes in nociception was beyond the scope of these particular studies.

The vIPAG is composed of diverse cell populations that express a number of different peptides, including CCK, GAL, SP, ENK, VIP, and SST along the rostrocaudal axis in the rat (Smith et al., 1994). A recent study reported that chemogenetic activation of GABAergic neurons in the vIPAG decreased thermal pain sensation, whereas chemogenetic inactivation of these cells increased thermal pain sensation (Samineni et al., 2017). Although this population comprises both SST- and PV-expressing interneurons, the SST-expressing subpopulation may be solely responsible for these effects, as optogenetic inhibition of these interneurons produced thermal analgesia, while optogenetic activation/inhibition of PV-expressing interneurons produced no changes in thermal pain sensitivity (N. Winke, INSERM, Neurocentre Magendie, U1215, personal communication). *Rxfp3* mRNA was co-expressed with *Sst* mRNA in ~7% of *Sst* mRNA-containing neurons in the vIPAG, making it unlikely that activation/inhibition of this RXFP3 population would elicit changes in pain behaviours. Nevertheless, this remains a relevant question to be addressed experimentally.

Chapter 5 – Anxiety-like behaviours in rat and mouse models of inflammatory pain

5.1 Introduction

A considerable amount of research has examined the interplay between chronic pain and anxiety disorders. Since the turn of the century, the number of studies focused on the comorbidity of anxiety and pain syndromes has steadily increased, making this currently a prominent topic in the field (Asmundson and Katz, 2009). Elucidating the mechanisms that underlie the relationship between these disorders requires an understanding of the mechanisms linked to the establishment of chronic pain, as well as comprehensive knowledge of the hodological composition of neural circuits that contribute to these phenomena. This Introduction provides an overview of the mechanisms through which long-lasting pain contributes to changes in neural circuits, as well as some key findings from human and animal studies that suggest long-lasting pain contributes to the expression of pain comorbidities, with a focus on data related to anxiety-related disorders. This Chapter comprises two studies that sought to (i) assess changes in anxiety-like behaviours in the CFA model of inflammatory pain in mice, and (ii) assess the effect of RXFP3 activation on anxiety-like behaviours in the CFA model in rats.

5.1.1 Synaptic plasticity

Synaptic plasticity refers to short- or long-lasting changes in synaptic strength. It is characterized by changes in synaptic transmission, conventionally measured using electrophysiological methods. Long-lasting changes in synaptic strength have been identified in several pain studies (**Table 5.1**).

Increased synaptic strength in the dorsal horn occurs in acute (Ikeda et al., 2006) and chronic (Sandkühler and Liu, 1998) pain states, suggesting that these changes begin during peripheral stimulation and last throughout pain ‘chronification’. As a result, in chronic pain, synaptic transmission is altered in projection neurons (Keller et al., 2007)

and supraspinal structures, including descending control pathways, where these changes alter pain perception by modulating the affective and sensory-discriminative components of pain.

Table 5.1. Synaptic plasticity in pain models.

Structure	Type of Pain	Model	Findings	Refs
Dorsal horn of spinal cord	Neuropathic	Sural nerve injury	Increased synaptic transmission of afferent fibre-spinal dorsal horn synapses	1
		CCI	Increased firing rate of layer I projection neurons	2
	Inflammatory	Capsaicin or Formalin	NMDAR- and NOS-dependent LTP in layer I projection neurons	3
Thalamus	Neuropathic	CCI	Increased synaptic transmission in neurons of the ventral posterolateral nucleus driven by Na _v 1.3	4
	Inflammatory	Carrageenan	Increased firing rate of ventrobasal neurons in response to noxious heat or pinching	5
Amygdala	Neuropathic	SNL	NMDAR-independent synaptic potentiation of BLA-CeA synapses	6
			Increased EPSCs in the CeA evoked by LPB stimulation	7
	Inflammatory	Kaolin-induced arthritis	Group I mGluR-dependent synaptic potentiation of BLA-CeA synapses	8
Insular cortex (IC)	Neuropathic	CPN ligation	Increase in AMPAR-mediated currents	8
Prefrontal cortex (PFC)	Neuropathic	SNI	Increase in NMDAR-, but not AMPAR-mediated transmission	10
Anterior cingulate cortex (ACC)	Neuropathic	CPN injury	Loss of presynaptic HCN channel-dependent LTP in thalamo-ACC synapses	11
	Inflammatory	CFA	BDNF-dependent increase in synaptic transmission	12
Primary and secondary sensory cortices (S1 and S2)	Inflammatory	CFA	Increase in AMPAR-mediated responses	13
			BDNF-dependent increase in synaptic transmission	12

1 – (Sandkühler and Liu, 1998); 2 – (Keller et al., 2007); 3 – (Ikeda et al., 2006); 4 – (Zhao et al., 2006); 5 – (Guilbaud et al., 1986); 6 – (Ikeda et al., 2007); 7 – (Nakao et al., 2012); 8 – (Neugebauer et al., 2003); 9 – (Qiu et al., 2014); 10 – (Metz et al., 2009); 11 – (Koga et al., 2015); 12 – (Thibault et al., 2014); 13 – (Eto et al., 2011).

Abbreviations: AMPAR – α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BDNF – brain-derived neurotrophic factor; BLA – basolateral amygdala; CCI – chronic constriction injury; CeA – central amygdala; CFA – complete Freund's adjuvant; CPN – common peroneal nerve; EPSC – excitatory postsynaptic current; HCN – hyperpolarization-activated cyclic nucleotide-gated; LPB – lateral parabrachial nucleus; LTP – long-term potentiation; NMDAR – N-methyl-D-aspartate receptor; NOS – nitric oxide synthase; PB – parabrachial nucleus; SNI – spinal nerve injury; SNL – spared nerve ligation.

5.1.2 Pain comorbidities

Long-lasting pain induces alterations in synaptic plasticity and circuitry that ultimately lead to maladaptive changes in cognition and affective behaviour. Among these, chronic pain is linked to increased incidences of depression, anxiety, and sleep disorders, which is in line with the idea that emotions play a role in the perception of pain (Roy et al., 2009).

In animal models, it has been widely demonstrated that stress plays a role in pain modulation. Acute stress induces analgesia in a manner that is temporally encoded by endogenous opioids such as ENK and β -endorphin (Parikh et al., 2011) and is modulated by several neurotransmitters, including serotonin (Coimbra and Brandão, 1997), GABA and glutamate (Butler and Finn, 2009). In contrast, chronic stress induces hyperalgesia (François et al., 2017; Jennings et al., 2014), reflecting the maladaptive nature of long-term changes in the circuitry.

Increased pain responses have been identified in animal models of depression (Li et al., 2016; Liu and Chen, 2014) and anxiety (Liu and Chen, 2014), and an increase in anxious behaviour has been observed in association with pain (François et al., 2017; Han

et al., 2015). Alterations in sleep patterns also affect pain thresholds: sleep restriction and sleep deprivation have been associated with increased sensitivity to pain in rodents and humans (Haack et al., 2007; Lautenbacher et al., 2006; Moldofsky, 2001). Conversely, decreased sleep efficiency has been described in pain models (Schütz et al., 2003). These findings suggest there is a reciprocal relationship between affective behaviours, sleep and pain, and is in line with findings in human studies (Asmundson and Katz, 2009; Moldofsky, 2001; Roy et al., 2009).

5.1.3 The pain/anxiety equation

Anxiety is a state of unpleasantness derived from expectation of a future threat (American Psychiatric Association, 2013). When considering its relationship to pain, anxiety is linked to both short- and long-lasting pain. In humans, expectation of painful heat stimuli was found to induce anxiety (Ploghaus et al., 2001), and anxiety is a comorbid state commonly associated with chronic pain (Asmundson and Katz, 2009). However, in rodent models of pain, anxiety-like behaviours are typically studied in the context of longer-lasting, chronic pain. In mice, CFA-induced inflammatory pain has been reported to increase anxiety-like behaviours on a timescale of weeks (Liu et al., 2015; Narita et al., 2006), but not days [see (Liu and Chen, 2014) for a summary of animal model findings]. Conversely, social defeat stress, used to induce elevated anxiety-like behaviours, has been found to induce hyperalgesia in rats (Andre et al., 2005) This is in line with findings that chronic stress exposure induces hyperalgesia, termed stress-induced hyperalgesia [see (Jennings et al., 2014) for review], and points towards a reciprocal relationship between pain and anxiety.

5.1.4 Specific aims

The experimental studies described in this Chapter, *(i)* determined whether persistent inflammatory pain induced changes in anxiety-like behaviours in mice, and *(ii)* assessed the effect of RXFP3 activation on anxiety-like behaviours in rats, under normal or inflammatory pain conditions.

5.2 Methods

5.2.1 Animals

Experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. All experiments were approved by the Animal Ethics Committee at The Florey Institute of Neuroscience and Mental Health (Ethics Numbers: 13-104 and 16-092). Experiments were performed using 8-13 week-old, male C57Bl6/J mice. Male Sprague-Dawley rats weighed 250-270 g upon arrival at the Animal Facility.

All animals had an acclimation period of 3-7 days upon arrival in the Animal Facility. Mice and rats were single-housed. For details on the lateral cerebral ventricle cannula implantation surgery in rats, the peptides and drugs injected, the saline and CFA injections, icv injections in rats, and cannula placement verification, please refer to Chapter 3, sections 3.2.2, 3.2.3, 3.2.4, 3.2.8, and 3.2.10, respectively. For the studies described, mice were injected with 50 μ L NaCl 0.9% or CFA, as indicated.

5.2.2 Behaviour testing

To test for changes in anxiety-like behaviours, mice and rats were submitted to the light-dark box (LDB) and elevated plus maze (EPM) tests. Mice were submitted to a baseline von Frey test (see section 3.2.5 for protocol) then injected with NaCl 0.9% or CFA into the right hindpaw, and tested for changes in PWT weekly for 4 weeks. Two cohorts were submitted to LDB and EPM testing: 12 mice (6 NaCl, 6 CFA) were tested 1 week after injection, whereas 20 mice (10 NaCl, 10 CFA) were tested 4 weeks after injection.

Rats received an icv injection 30 min prior to being placed into the LDB or EPM at the beginning of the experiment. This cohort comprised rats that had been injected with NaCl or CFA 3 weeks prior to testing, as in Chapter 3 (see section 3.3.2 for von Frey results).

5.2.3 Light-dark box (LDB)

Mice were placed into a 27.5 × 27.5 cm locomotor cell (MedAssociates, St. Albans, VT, USA) with a semi-opaque black box insert in half of the cell. Rats were placed in the 42 cm (length) × 42 cm (width) × 40 cm (height) automated locomotor cell (MedAssociates). A small opening enabled animals to enter or leave the dark compartment. The light side was lit by an array of light-emitting diodes (600 lx in the centre for mice, 400 lx for rats), creating an aversive stimulus. Horizontal and vertical movements (rearing) were tracked using Activity Monitor V6.02 software (MedAssociates). Sessions lasted 10 min and boxes were cleaned with ethanol 70%v/v and dried between experiments. Parameters measured included number of entries into the light compartment, time spent in light compartment, number of transitions to the light compartment and latency to enter the light compartment. Animals that did not enter the light compartment during the test were excluded, which resulted in 5 rats being excluded from both vehicle- and agonist-treated groups. No mice were excluded.

5.2.4 Elevated plus maze (EPM)

The EPM consisted of an opaque grey acrylic material in a plus-sign shape (mice: 30 cm above ground, with four 4 cm wide, 30 cm long arms, 20 cm high walls in the closed arms; rats: 72 cm above the ground, 12 cm wide, 40 cm long arms, 40 cm high walls in the closed arms). Each animal was placed in the centre square (5 × 5 cm for mice, 12 × 12 cm for rats) of the maze at the start of recording. Each trial ran for 10 min and

movements, including duration and entries into the open and closed arms were tracked using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands). Parameters measured included time spent in open/closed arms and distance in open/closed arms. Animals that did not enter the open arms during the test were excluded, which involved one rat, and 3 mice from saline- and CFA-injected groups being excluded.

5.2.5 Statistical analysis

Data was analysed using GraphPad Prism 7.0. Results are expressed as mean \pm S.E.M. For the mouse experiments, LDB and EPM data were compared using unpaired t-test to test for the effect of 'injection' (NaCl vs CFA). Von Frey data were compared using one-way ANOVA with time as a 'within' factor, followed by Sidak's multiple comparison test to assess the effect of injection. For rat experiments, the Kruskal-Wallis test was used to assess the effect of 'injection' (NaCl vs CFA) or 'treatment' (vehicle vs agonist), followed by Dunn's multiple comparison *post-hoc* test to assess any interaction between injection and treatment. Statistical significance was fixed at $p < 0.05$.

5.3 Results

5.3.1 Anxiety-like behaviours in mice with inflammatory pain

To assess whether mechanical pain thresholds were altered over a 4-week testing period, mice were submitted to von Frey testing once a week (**Figure 5.1**). Mice injected with CFA (n = 10) displayed decreased PWT that persisted over 4 weeks, suggesting there are no significant changes in mechanical pain thresholds within this time window.

Given that mice injected with CFA display decreased PWT over a broad time window, possible changes in anxiety-like behaviours resulting from prolonged sensitization of peripheral nociceptors were assessed in the LDB and EPM tests. There were no significant differences between mice injected with saline or CFA in the time spent in the light compartment of the LDB during the first or second 5 min of the test, both 1 week and 4 weeks after saline/CFA injection (**Figure 5.2**). Additionally, there were no significant differences in the time spent in the open arms of the EPM during the first or second 5 min of the test at either of the test time points (**Figure 5.3**). Overall, there were no significant differences between saline- or CFA-injected mice in any of the assessed parameters (Appendix 2). These results suggest that mice with persistent inflammatory pain do not express a strong anxiety-like phenotype.

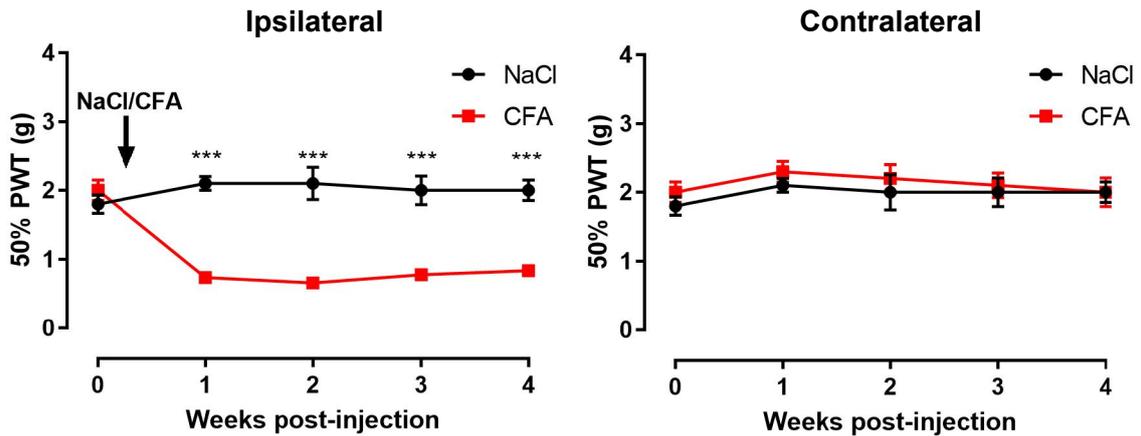


Figure 5.1. CFA-induced decrease in mechanical pain thresholds persisted over 4 weeks. Mice were subjected to von Frey testing to ascertain changes in mechanical pain thresholds, represented as PWT, over 4 weeks. Mice injected with saline (NaCl, black lines and circles, n = 10) displayed no changes in mechanical pain thresholds, whereas mice injected with CFA (red lines and squares, n = 10) displayed decreased PWT in the ipsilateral paw in all cohorts when compared to week 0. Conversely, there was no change in PWT in either group over the 4-week period in the contralateral paw. I.pl. injection of saline or CFA is indicated after week 0 by a black arrow and the heading ‘NaCl’ or ‘CFA’, respectively. Results for the ipsi- and contralateral paws are indicated with the respective heading. ***p<0.001 using one-way ANOVA followed by Sidak’s multiple comparison test for the effect of injection.

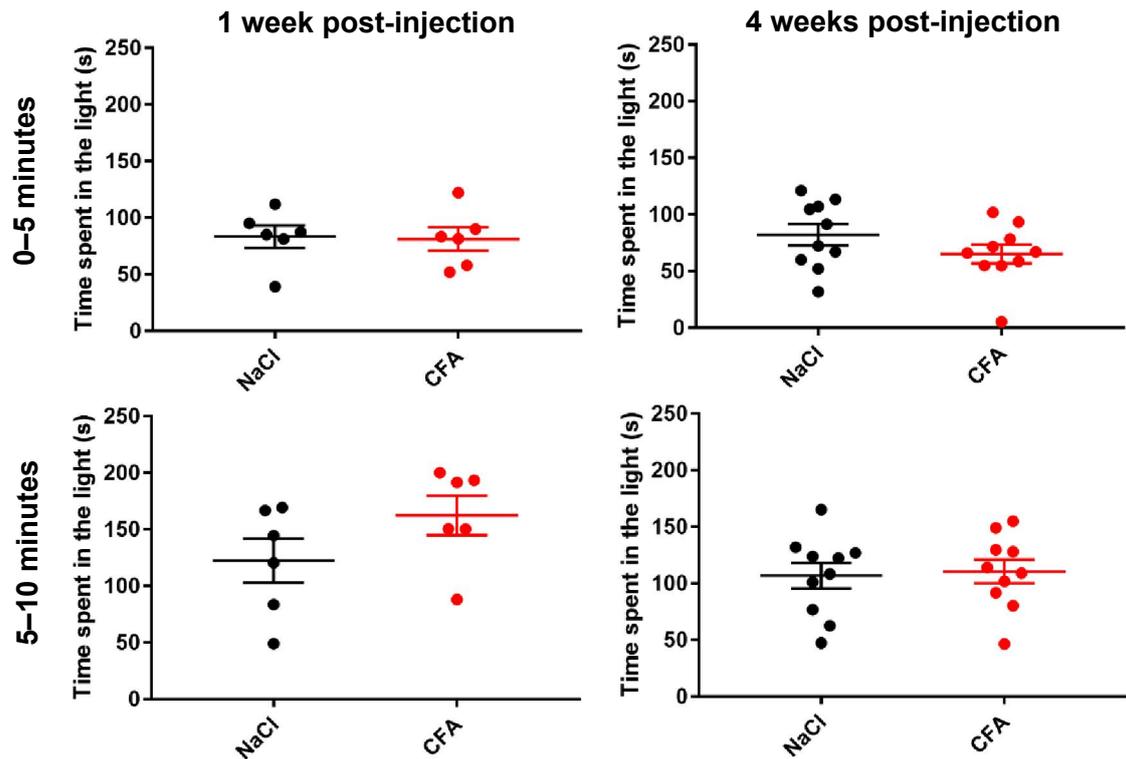


Figure 5.2. Persistent inflammatory pain does not induce changes in anxiety-like behaviour in the LDB. Mice were subjected to the LDB test to ascertain changes in anxiety-like behaviours, represented as time spent in the light compartment, in the first 5 min (top graphs, labelled '0-5 minutes') or second 5 min (bottom graphs, labelled '5-10 minutes'), at 1 week (left, n = 6) or 4 weeks (right, n = 10) after i.pl. injection of saline or CFA (black or red dots, as indicated below). No significant differences were found using unpaired non-parametric Mann-Whitney test.

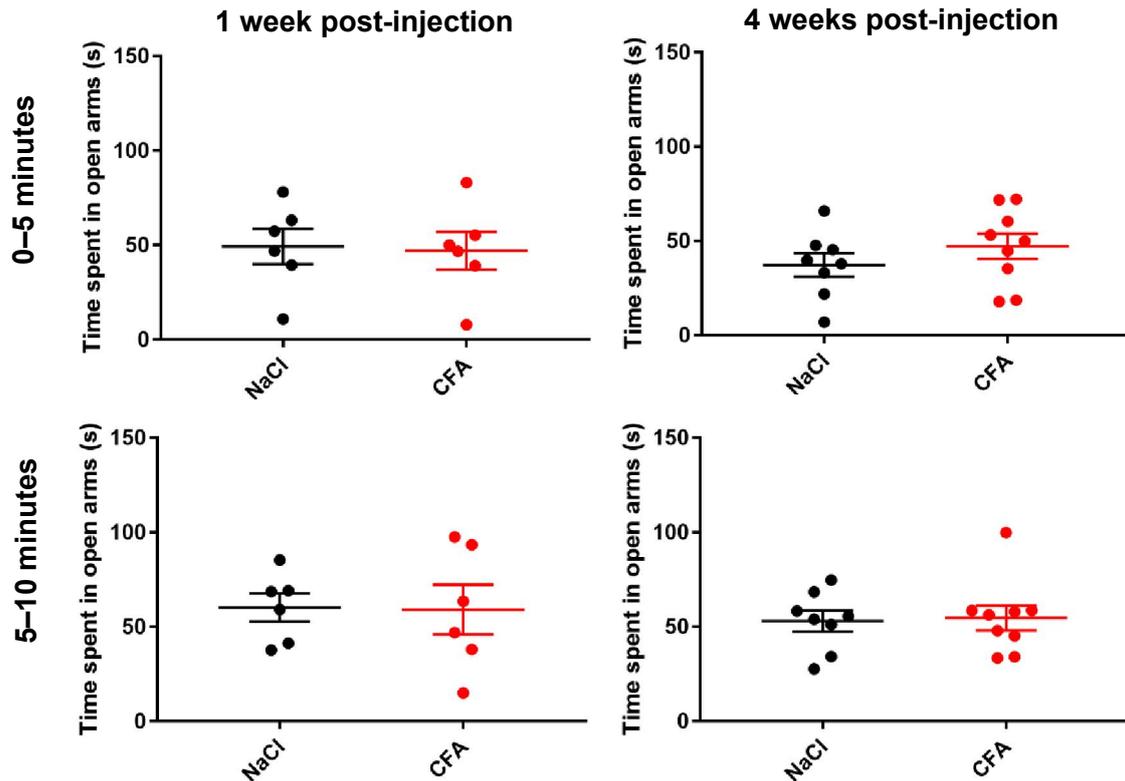


Figure 5.3. Persistent inflammatory pain does not induce changes in anxiety-like behaviours in the EPM. Mice were subjected to the EPM test to ascertain changes in anxiety-like behaviours, represented as time spent in the open arms of the maze, in the first 5 min (top graphs, labelled ‘0-5 minutes’ on the left) or second 5 min (bottom graphs, labelled ‘5-10 minutes’), at 1 week (left, n = 6) or 4 weeks (right, n = 8 saline, n = 9 CFA) after i.pl. injection of saline or CFA (black or red dots, as indicated below). No significant differences were found using unpaired non-parametric Mann-Whitney test.

5.3.2 Pharmacological studies in rats

To assess the effect of RXFP3 activation on anxiety-like behaviours under inflammatory pain conditions, rats that had been injected with 0.9% NaCl or CFA were treated with aCSF or RXFP3-A2 30 min before being subjected to the LDB or EPM tests. There were no significant differences in time spent in the light compartment of the LDB between the different groups in the first and second 5 min of the test (**Figure 5.4**). There were no significant differences in time spent in the open arms of the EPM between the different groups in the first 5 min of the test (**Figure 5.5**). There was a significant decrease in time spent in the open arms in rats injected with CFA in the second 5 min of the test ($p < 0.05$), but there were no significant differences between saline- and RXFP3-A2-treated groups. No significant differences were detected in the other measured parameters (**Appendix 3**). These results suggest that CFA treatment can increase anxiety-like behaviours in rats, consistent with the idea that persistent inflammatory pain can induce a mild anxious-like phenotype.

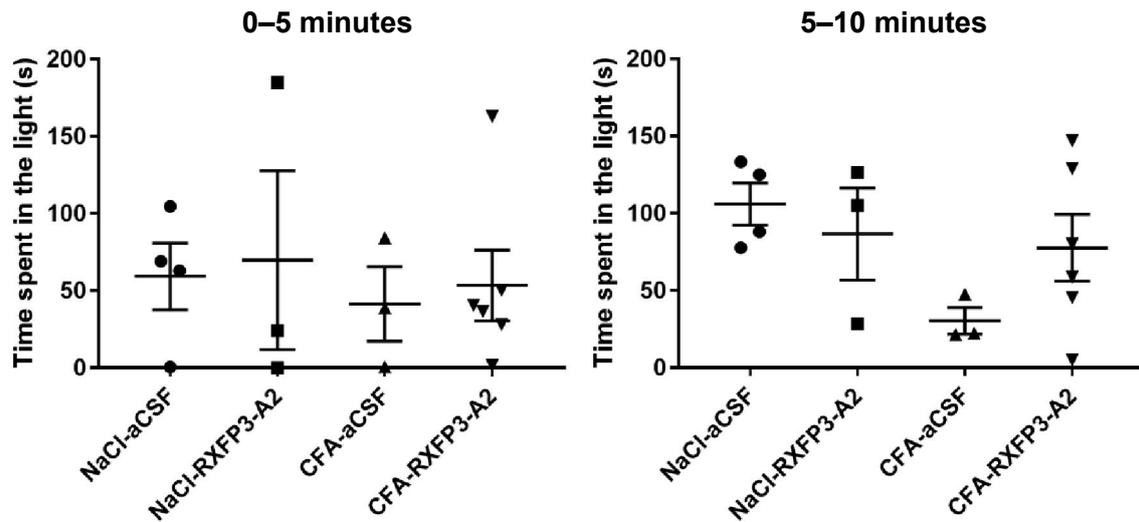


Figure 5.4. RXFP3 activation does not alter anxiety-like behaviours in rats under normal or inflammatory pain conditions in the LDB. Rats were treated with aCSF (n = 4 NaCl, n = 3 CFA) or RXFP3-A2 (n = 3 NaCl, n = 6 CFA) 30 min before being subjected to the LDB test to ascertain changes in anxiety-like behaviours, reflected by the time spent in the light compartment in the first 5 min (left, ‘0-5 minutes’) and second 5 min (right, ‘5-10 minutes’). No significant differences were found using Kruskal-Wallis test followed by Dunn’s multiple comparison test.

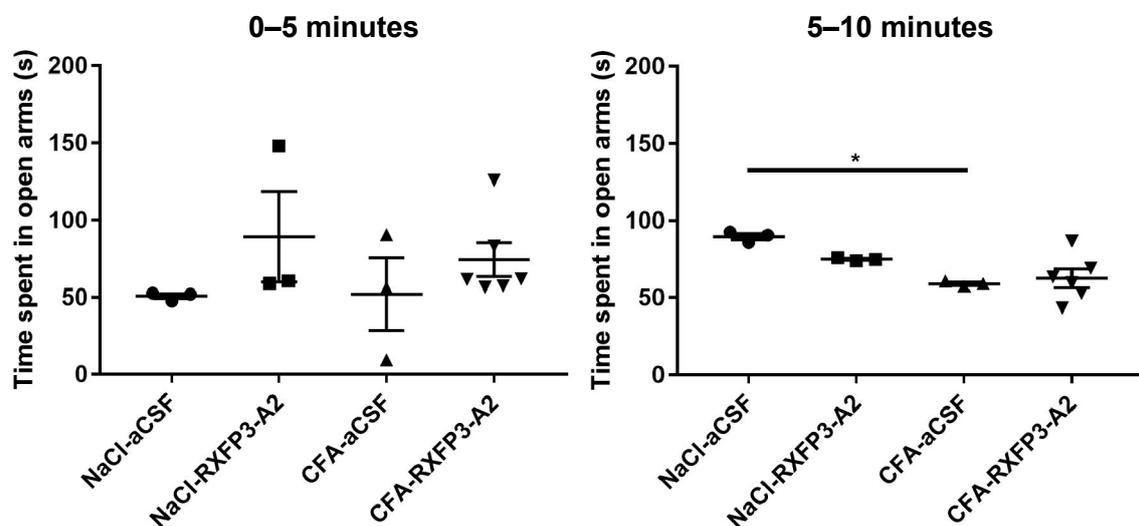


Figure 5.5. RXFP3 activation does not alter anxiety-like behaviours in rats under normal or inflammatory pain conditions in the EPM. Rats were treated with aCSF (n = 3 NaCl, n = 3 CFA) or RXFP3-A2 (n = 3 NaCl, n = 6 CFA) 30 min before being subjected to the EPM test to ascertain changes in anxiety-like behaviours, reflected by the time spent in the light compartment in the first 5 min (left, ‘0-5 minutes’) and second 5 min (right, ‘5-10 minutes’). * $p < 0.05$ using Kruskal-Wallis test followed by Dunn’s multiple comparison test.

5.4 Discussion

These studies assessed the possible presence of an anxiety-like phenotype in mice and rats with persistent hindpaw inflammatory pain. The results obtained suggest inflammatory pain does not induce strong changes in anxiety-like behaviours in mice, while it has a mild anxiogenic effect in rats. Moreover, studies in rats to assess the effect of central RXFP3 activation on anxiety-like behaviours found no effect of an icv administration of the agonist peptide, RXFP3-A2 on anxiety-like parameters.

While these data can be taken on face value, there are, in this case, some confounding factors associated with the study design that may have influenced the outcomes. A potentially important factor in both studies was that the animals were single-housed throughout, which can lead to social isolation and related behavioural changes. For example, social isolation is a known model of aggression in rodents, and it increases anxiety-like behaviours in mice (Ieraci et al., 2016) and rats (Djordjevic et al., 2012). It is possible that in these studies social isolation may have played a role in generating a ‘ceiling effect’, whereby inflammatory pain was not able to produce an increase in anxiety-like behaviours above a potentially already high level.

The mouse studies were designed to assess the presence of an anxiety-like phenotype under inflammatory pain conditions. To do so, experimental conditions that had previously shown to elevate anxiety-like behaviours were used (Narita et al., 2006). Despite using mice of the same strain, age, and sex, and similar doses of saline and CFA, the current studies did not replicate the results of the earlier study (Narita et al., 2006). A key difference in study design between the two studies is that, in the present experiments, mice were handled daily before being tested in the LDB and EPM, which presumably limits avoidance to the experimenter. Moreover, luminosity in the LDB was higher in the present study (600 lx vs 500 lx), which may trigger an over-aversion to the light

compartment. Together, these or other less defined factors may have masked an anxiety-like phenotype in the current study and underlie the study differences.

Lastly, the pharmacological studies in rats shared a similar design to those completed earlier in the host laboratory, in which the effect of RXFP3 activation on *basal* anxiety levels in rats was assessed (Philip J. Ryan et al., 2013). In that report, icv administration of RXFP3-A2 produced a decrease in anxiety-like behaviours in the rat LDB and EPM, but ‘basal’ levels of anxiety were quite high in this study (Philip J. Ryan et al., 2013). Nonetheless, the present studies did not replicate these findings, as the results indicated that RXFP3 activation did not alter anxiety-like behaviours in rats under normal pain conditions. The timing of the different anxiety-like behaviour assays post-treatment was 30 min (current study) and 10 min (Philip J. Ryan et al., 2013), which may have reduced the ability of agonist treatment to produce significant changes in anxiety-like behaviours in the present study, if the peptide is rapidly inactivated. Another possible weakness of the current study is that the final sample size may have been too small to provide sufficient statistical power in the behavioural assays. Therefore, future studies should consider where possible, the use of larger cohorts of group-housed animals, as well as other changes in the study design.

Chapter 6 – General Discussion

6.1 Summary of main findings

These studies are the first to implicate the RLN3/RXFP3 system in the modulation of nociception in the mouse. The main finding of the pharmacological studies in mice was that central RXFP3 activation via icv administration of a selective peptide agonist produced mechanical, but not thermal, analgesia, and that, based on studies of the effect of an RXFP3 antagonist, tonic endogenous RLN3 signalling affects normal pain thresholds for both modalities. Furthermore, IHC studies in mice treated with an RXFP3 agonist peptide that used Fos-immunostaining as a surrogate or marker for neuronal activity patterns, sought to identify the brain areas involved in RXFP3 modulation of pain. Despite ISH studies indicating the presence of *Rxfp3* mRNA in discrete neuronal subpopulations in these areas, the completed IHC studies were not able to confirm a specific brain area(s) involved in the observed pharmacological effects (see section 4.4 for details). Quantitative studies of anxiety-like behaviours indicated that inflammatory hindpaw pain in mice did not correlate with comorbid anxiety, suggesting that changes in nociceptive thresholds elicited by RXFP3 activation are more specifically related to pain-related circuits under the experimental conditions employed.

6.2 Technical considerations

The studies described in this thesis employed an inflammatory pain model, in which CFA was injected into the right hindpaw of adult WT or RXFP3 KO mice and adult WT rats. This approach elicits long-lasting changes in pain thresholds lasting at least 4 weeks in mice (see section 5.3.1). This allowed for the study of the effects of RXFP3 activation on pain thresholds in the acute and chronic stages of inflammatory pain. However, there are limitations to the use of this model. Firstly, it does not allow for the study of the role of the RLN3/RXFP3 system in pain onset, as changes to nociceptive thresholds occur within

2 h of CFA injection (Ren and Dubner, 1999). This greatly reduces the time window for studying the effect of pharmacological interventions targeting the RLN3/RXFP3 system that could potentially limit the expression of allodynia post-injury. This problem could be overcome by using pain models in which the time between injury and onset of allodynia is longer (e.g. neuropathic or cancer-induced pain models). Secondly, this model does not allow the assessment of the role of the RLN3/RXFP3 system on the aversive component of pain. Although these studies focused on the sensory component of pain, aversion also plays an important role in nociception, especially in perpetuating sensitization of peripheral nociceptors (Fields, 1999). In keeping with this, another limitation of the use of the CFA model is that it does not allow differentiation between the contribution of peripheral and central sensitization, which depend on the involvement of distinct sensory fibres and neural circuits (Latremoliere and Woolf, 2009). As such, it is not possible to assess the involvement of the RLN3/RXFP3 system on these processes. However, these two issues can be tackled using a formalin-induced pain model, albeit in separate contexts. Formalin induces two separate phases of allodynia, with the first involving direct activation of peripheral nociceptors, and the second involving the establishment of central sensitization (Ren and Dubner, 1999). In addition to this, because of its short-lasting effects (pain thresholds recover ~1 h after injection), formalin is the drug of choice for studying the aversive component of pain, as it can induce CPA, which involves repeated reinforcement of a pain/location dichotomy, to study avoidance behaviour in the absence of a painful stimulus. Thus, this would be the preferred model for studying the involvement of the RLN3/RXFP3 system in the aversive component of pain, as well as its role in peripheral and central sensitization.

6.3 Future directions

The present studies implicated the RLN3/RXFP3 system in nociception in a model of persistent inflammatory pain, but there are still numerous questions that remain answered. As discussed, the pharmacological studies in Chapter 3 focused on the sensory-discriminative component of pain, but failed to test whether the RLN3/RXFP3 system modulates the aversive component of pain. This issue should be addressed in future experiments in experiments looking into pain-induced CPA.

Considering the pharmacological studies in mice and rats, and previous experiments performed in rats (S. Ma and K. Egron, unpublished data, section 3.1.2), they suggest that activation of RXFP3 produces analgesia in the initial (acute) stages of inflammatory pain. As such, future studies should explore the effects of RXFP3 activation in models of acute inflammatory pain, such as formalin- and carrageenan-induced inflammation. Moreover, the present studies revealed that activation of RXFP3 produces mechanical, but not thermal, analgesia in inflammatory pain conditions. Thus, a similar design should be used to assess the effects of RXFP3 activation in pain models that produce mechanical, but not thermal, allodynia, as is the case for the Seltzer (sciatic) and spared nerve injury models (Shields et al., 2003).

Another remaining issue is the identification of brain areas that are involved in the observed effects. Although the completed IHC studies sought to identify the target regions for the effects of RXFP3 activation on nociception, it was not possible to clearly discern one or more relevant target. In addition to the suggested changes in the experimental design (section 4.4), it would be interesting to study the effects of RXFP3 activation through localized injection of peptide agonist into candidate brain areas, as performed in the present studies for the ACC (section 3.3.1). Moreover, assessing the

specific neuronal sub-populations involved in the observed effects would provide greater insight into the hodological and cellular composition of these neural circuits.

Finally, given the involvement of the RLN3/RXFP3 system in stress responsiveness (see section 3.1.2) and the reciprocal relationship between stress and pain, it would be interesting to explore the effects of RXFP3 activation/inhibition on nociception in experiments that combine the two elements. These experiments could include the study of such effects on latent sensitization, in which post-injury recovery of normal pain thresholds can be ablated by exposure to stress (see section 3.4 for further details). Another possibility is the use of models that produce simultaneous changes in pain and stress levels, including models of stress-induced hyperalgesia (SIH) (Jennings et al., 2014) and analgesia (SIA) (Butler and Finn, 2009), as well as combinations of injury-induced pain (e.g. CFA-induced inflammation) with stress exposure (e.g. swim stress).

6.4 Conclusions

Understanding the mechanisms involved in nociception and how diverse neuropeptide systems modulate pain thresholds is at the forefront of research into drug development. While the present studies provide the first evidence that the RLN3/RXFP3 system is involved in regulation of nociceptive thresholds, further studies are warranted to clarify the mechanisms, pain modalities, and time window in which RXFP3 activation can effectively produce analgesia. Furthermore, the involvement of this neuropeptide system in pain-related behaviours, such as sleep, anxiety, and depression, suggests it may be a suitable target for development of new therapeutics that reduce pain and dampen the expression/progression of comorbid disorders. The development of new pharmacological therapies requires the continued study of the RLN3/RXFP3 system and others, which can potentially improve the quality-of-life of individuals burdened by chronic pain.

Appendix

Appendix 1

Table A.1. Comparison of total number of Fos-IR-positive cells by brain area

Brain area	Condition	n	SR	C	CR	p-value
ACC	NaCl/aCSF	3	12	3	[0] U [9]	0.700
	NaCl/RXFP3-A2	3	9			
amBNST	NaCl/aCSF	3	15	0	[0] U [9]	N/A
	NaCl/RXFP3-A2	3	6			
PT	NaCl/aCSF	4	14	4	[0-1[U]11-12]	0.629
	NaCl/RXFP3-A2	3	14			
LA	NaCl/aCSF	4	12	6	[0-1[U]11-12]	0.990
	NaCl/RXFP3-A2	3	16			
BLA	NaCl/aCSF	4	15	3	[0-1[U]11-12]	0.400
	NaCl/RXFP3-A2	3	13			
CeA	NaCl/aCSF	4	14	4	[0-1[U]11-12]	0.629
	NaCl/RXFP3-A2	3	14			
ACC	CFA/aCSF	3	15	9	[0-1[U]14-15]	0.786
	CFA/RXFP3-A2	5	21			
amBNST	CFA/aCSF	3	20	14	[0-1[U]14-15]	0.071
	CFA/RXFP3-A2	5	16			
PT	CFA/aCSF	3	12	6	[0-1[U]14-15]	0.786
	CFA/RXFP3-A2	5	24			
LA	CFA/aCSF	3	15	9	[0-1[U]14-15]	0.786
	CFA/RXFP3-A2	5	21			
BLA	CFA/aCSF	3	15	9	[0-1[U]14-15]	0.786
	CFA/RXFP3-A2	5	21			
CeA	CFA/aCSF	3	17	11	[0-1[U]14-15]	0.393
	CFA/RXFP3-A2	5	19			
ACC	NaCl/aCSF	3	10	5	[0] U]8-9]	0.629
	CFA/aCSF	3	11			
amBNST	NaCl/aCSF	3	9	6	[0] U]8-9]	0.700
	CFA/aCSF	3	12			
PT	NaCl/aCSF	4	16	6	[0-1[U]11-12]	0.990
	CFA/aCSF	3	12			
LA	NaCl/aCSF	4	16	6	[0-1[U]11-12]	0.990
	CFA/aCSF	3	12			
BLA	NaCl/aCSF	4	15	7	[0-1[U]11-12]	0.857
	CFA/aCSF	3	13			
CeA	NaCl/aCSF	4	11	11	[0-1[U]11-12]	0.114
	CFA/aCSF	3	17			

(continued next page)

Table A.1 (continued)

ACC	NaCl/RXFP3-A2	3	11	13	[0-2[U]13-15]	0.571
	CFA/RXFP3-A2	5	25			
amBNST	NaCl/RXFP3-A2	3	17	13	[0-2[U]13-15]	0.393
	CFA/RXFP3-A2	5	19			
PT	NaCl/RXFP3-A2	3	14	10	[0-2[U]13-15]	0.990
	CFA/RXFP3-A2	5	22			
LA	NaCl/RXFP3-A2	3	19	8	[0-2[U]13-15]	0.143
	CFA/RXFP3-A2	5	17			
BLA	NaCl/RXFP3-A2	3	16	9	[0-2[U]13-15]	0.571
	CFA/RXFP3-A2	5	20			
CeA	NaCl/RXFP3-A2	3	14	7	[0-2[U]13-15]	0.990
	CFA/RXFP3-A2	5	22			

C – test result; CR – critical region; n – number of mice; N/A – cannot be calculated; SR – sum of ranks

Appendix 2

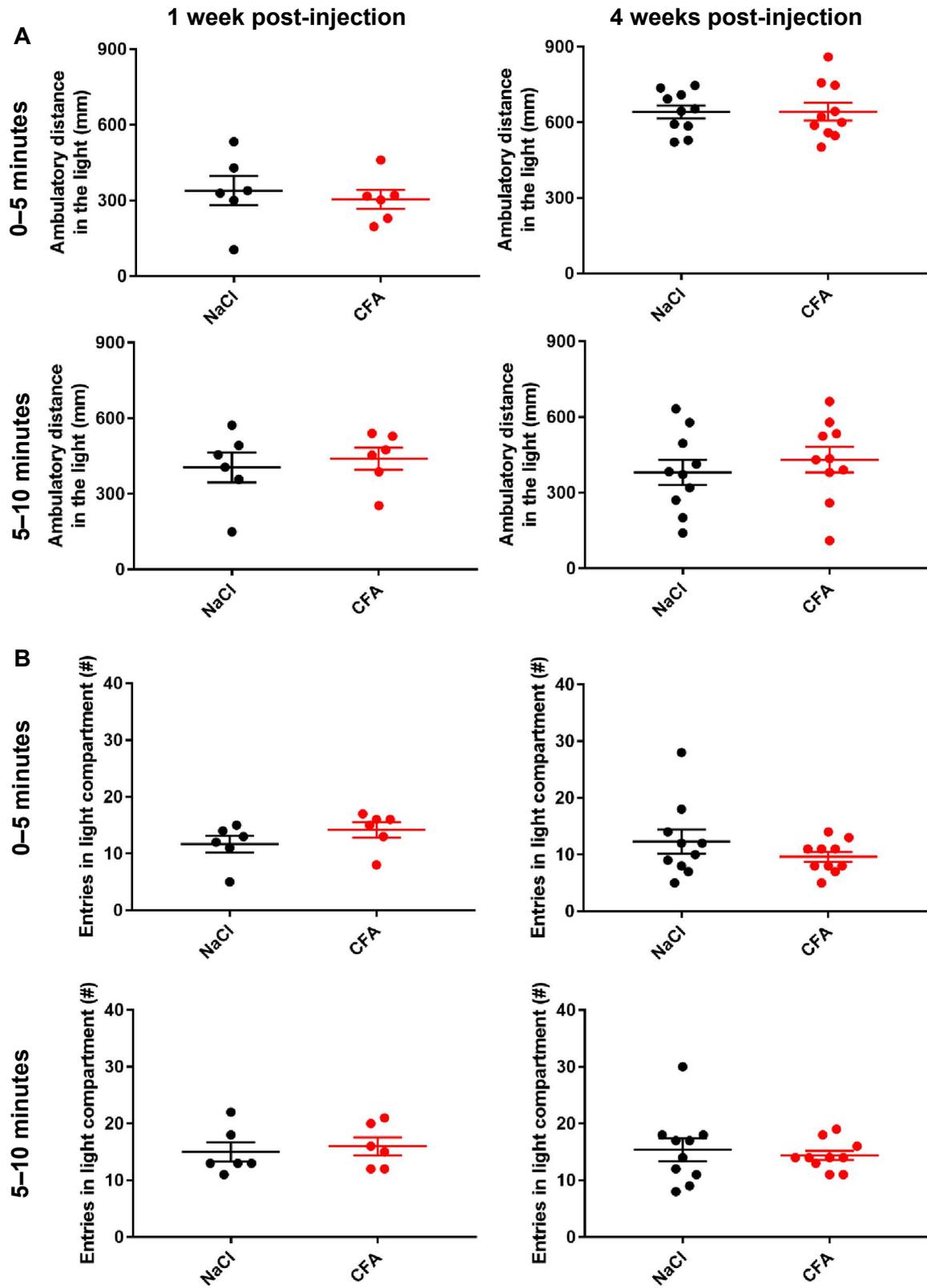
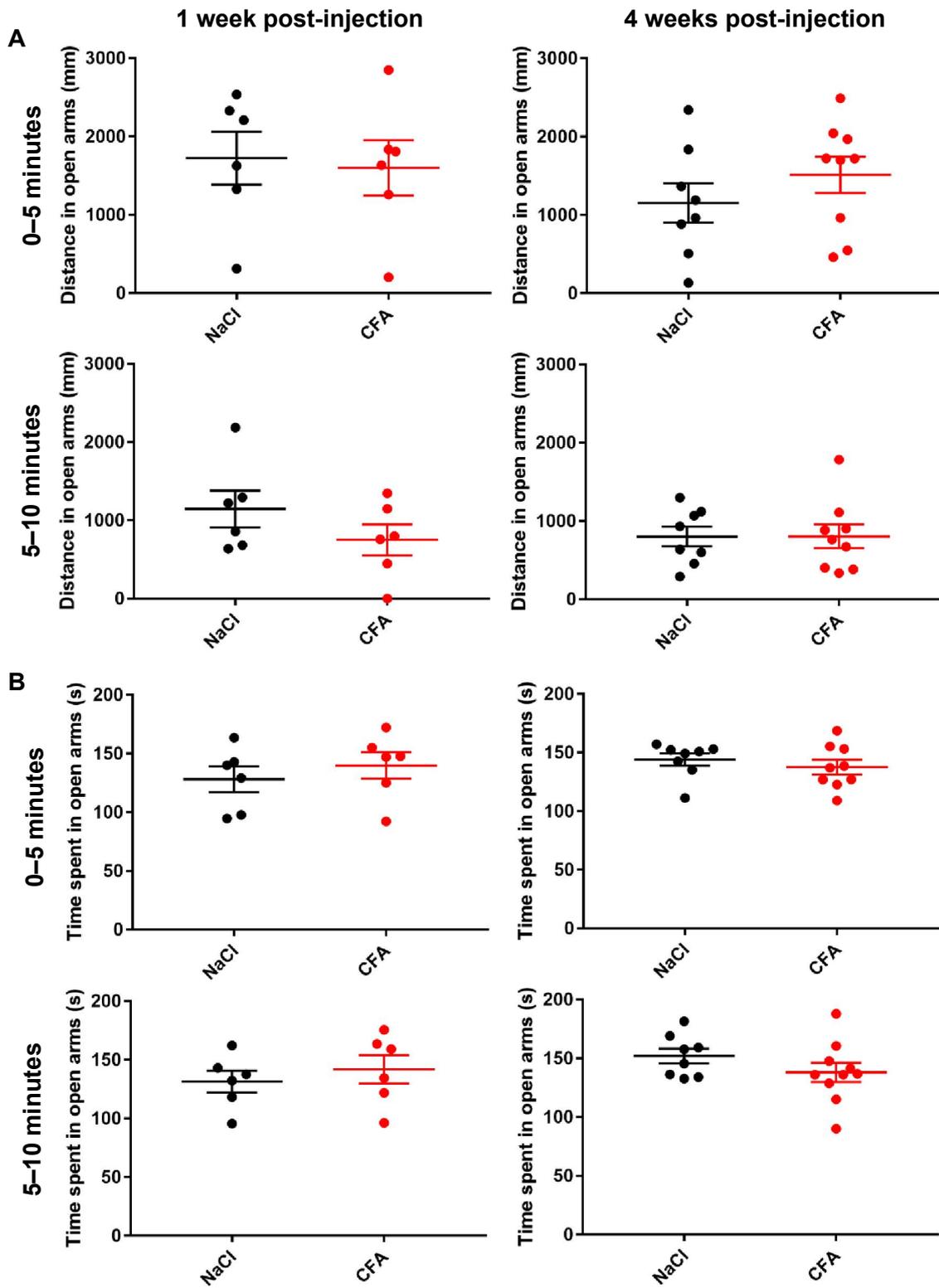


Figure A.1. Persistent inflammatory pain in mice did not alter ambulatory distance or number of entries into the light compartment of the LDB. (A) Distance travelled within the light compartment and (B) number of entries into the light compartment were measured to assess changes in anxiety-like behaviours. Parameters were measured in the first 5 min (top graphs, labelled '0-5 minutes' on the left) and second 5 min (bottom graphs, labelled '5-10 minutes', left), at 1 week (left, n = 6) or 4 weeks (right, n = 10) after the injection of saline or CFA (black and red symbols, respectively). No significant differences were found using unpaired non-parametric Mann-Whitney test.



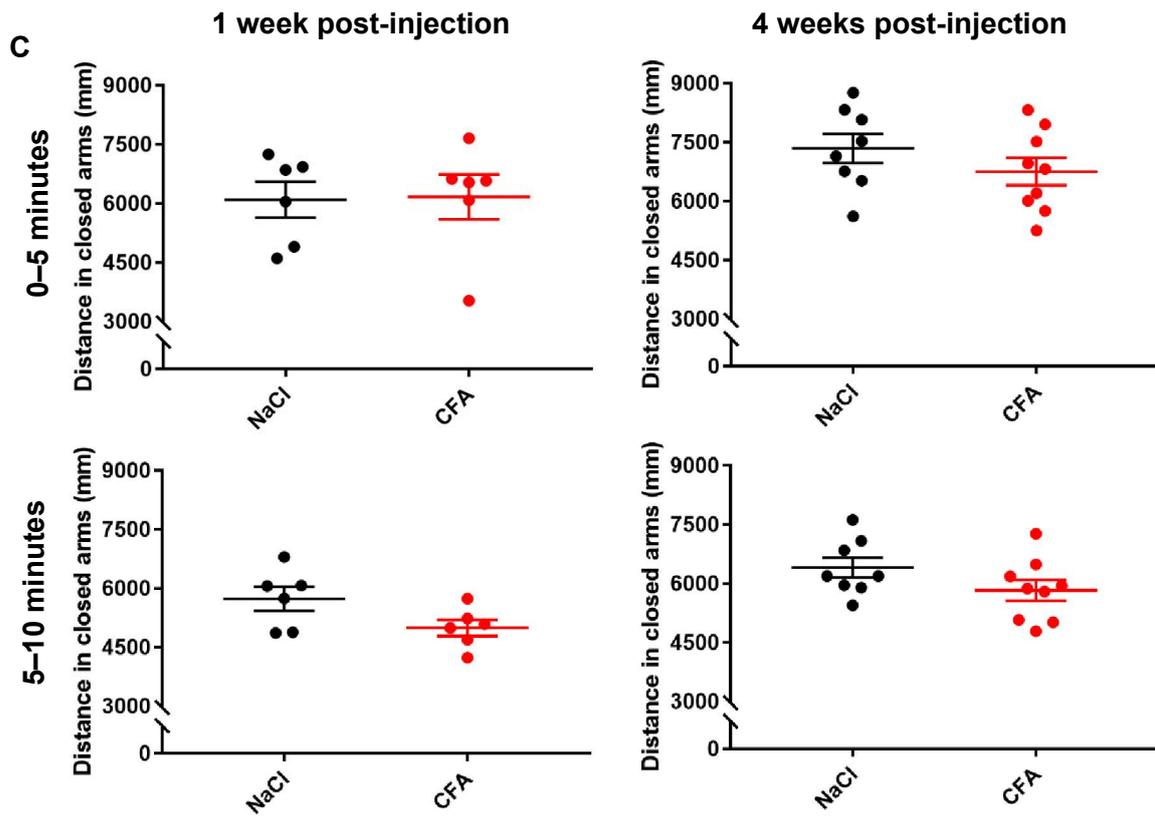


Figure A.2. Persistent inflammatory pain in mice did not alter ambulatory distance travelled in the arms or time spent in the closed arms of the EPM. (A) Distance travelled in the open arms, (B) time spent in the closed arms, and (C) distance travelled in the closed arms were measured to assess changes in anxiety-like behaviours. Parameters were measured in the first 5 min (top graphs, labelled ‘0-5 minutes’) and second 5 min (bottom graphs, labelled ‘5-10 minutes’), at 1 week (left, n = 6) or 4 weeks (right, n = 8 saline, n = 9 CFA) after the injection of saline or CFA (black and red symbols, respectively). No significant differences were found using unpaired non-parametric Mann-Whitney test.

Appendix 3

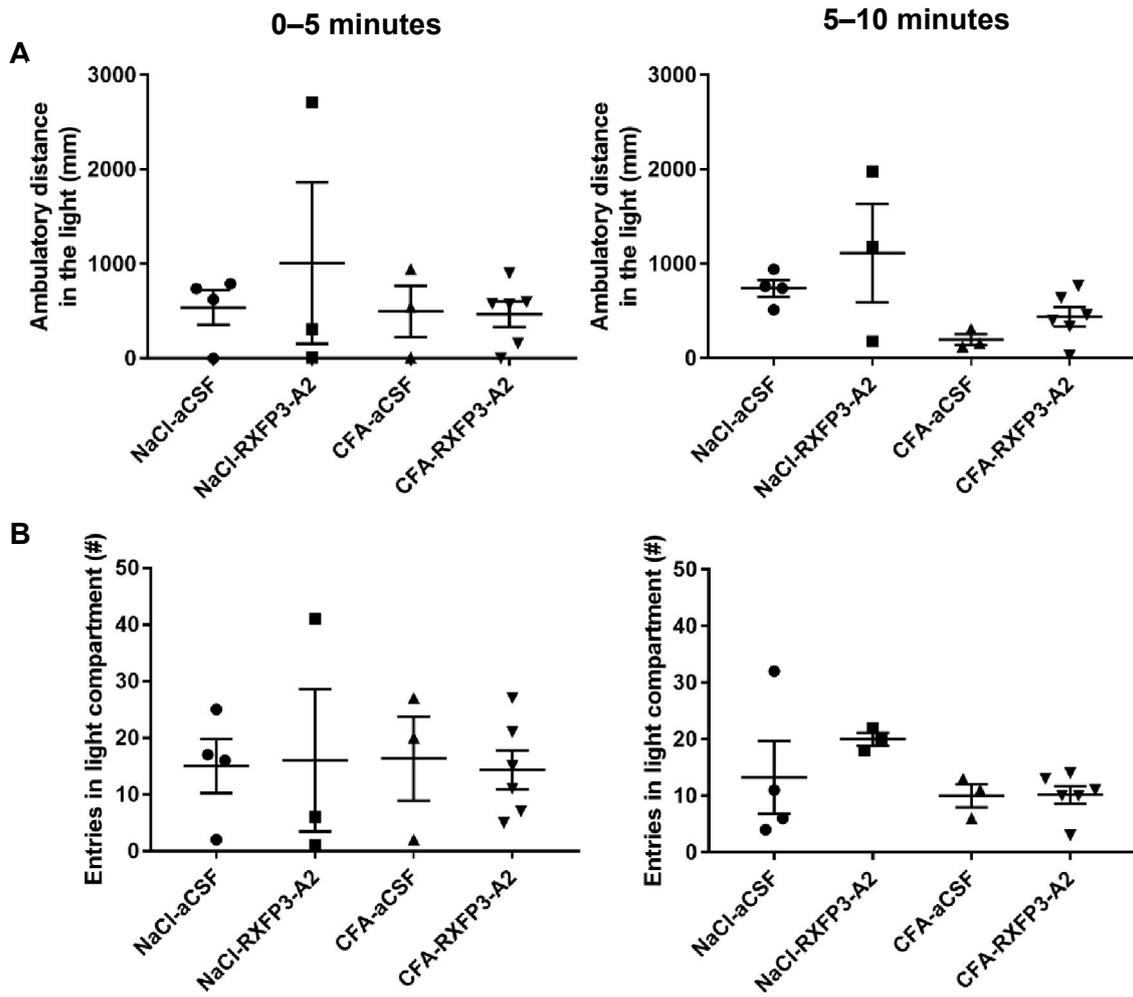


Figure A.2. Persistent inflammatory pain in mice did not alter ambulatory distance travelled in the arms or time spent in the closed arms of the EPM. (A) Distance travelled in the open arms, (B) time spent in the closed arms, and (C) distance travelled in the closed arms were measured to assess changes in anxiety-like behaviours. Parameters were measured in the first 5 min (top graphs, labelled ‘0-5 minutes’) and second 5 min (bottom graphs, labelled ‘5-10 minutes’), at 1 week (left, $n = 6$) or 4 weeks (right, $n = 8$ saline, $n = 9$ CFA) after the injection of saline or CFA (black and red symbols, respectively). No significant differences were found using unpaired non-parametric Mann-Whitney test.

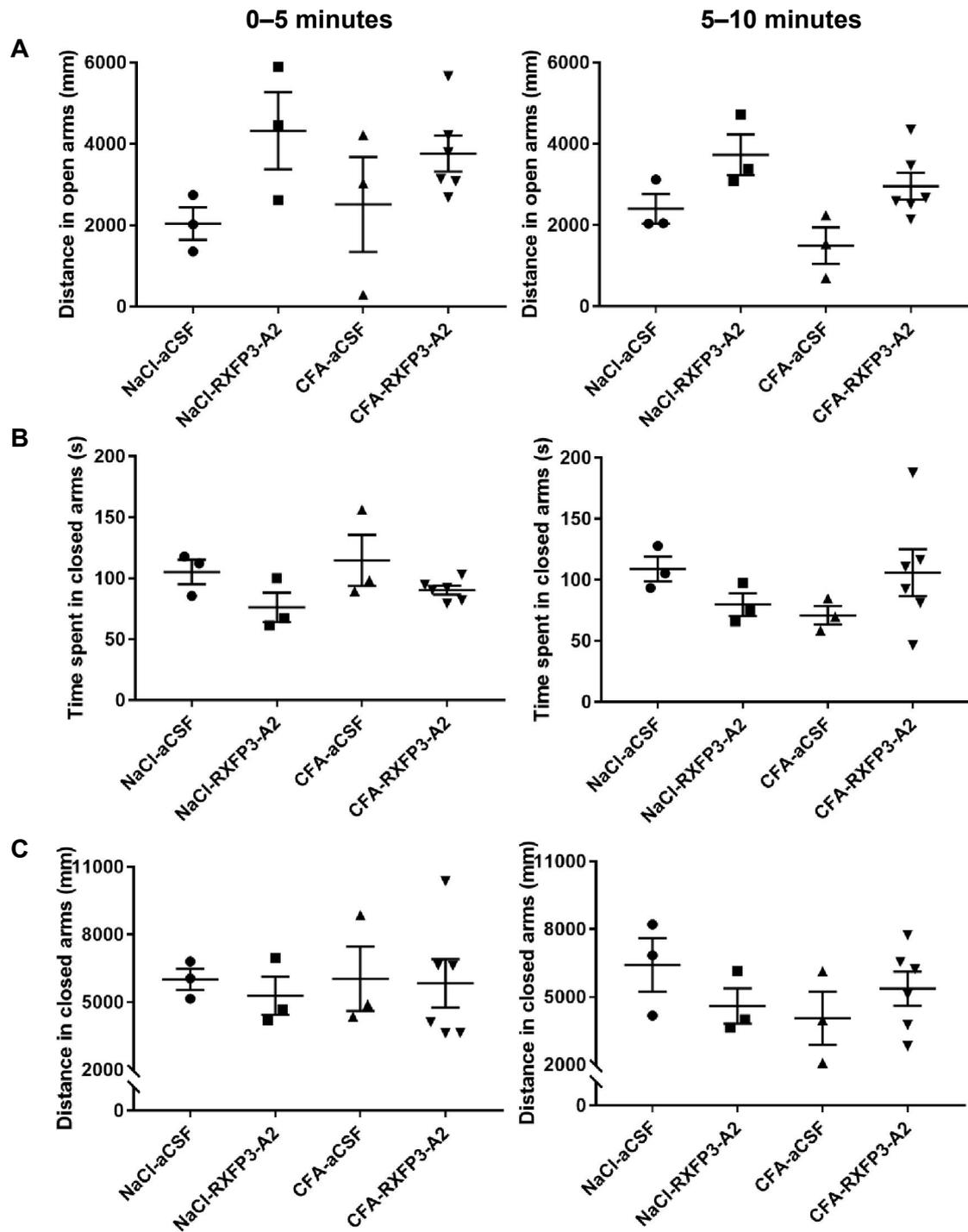


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