The role of the endogenous opioid system in hyperalgesia associated with negative affect

Mehnaz Islam Ferdousi, M.Sc.

Pharmacology and Therapeutics, School of Medicine

NCBES Galway Neuroscience Centre and Centre for Pain Research

National University of Ireland Galway

Supervisors:

Professor David P Finn
Pharmacology and Therapeutics, School of Medicine, National University of Ireland Galway

Professor John P Kelly
Pharmacology and Therapeutics, School of Medicine, National University of Ireland Galway

Dr Michelle Roche
Physiology, School of Medicine, National University of Ireland Galway

A thesis submitted to the National University of Ireland Galway for the degree of Doctor of Philosophy

February 2020

Table of contents

Abstract

Pain and negative affect (anxiety and depression) modulate one another reciprocally. However, the neurobiological mechanisms underlying this interaction are unclear. The endogenous opioid system plays a key role in regulating pain and affect but its role in pain-negative affect interactions, and the influence of genetic background thereon, is poorly understood. The inbred Wistar-Kyoto (WKY) rat, a genetic model of anxiety and depression, displays increased sensitivity to noxious stimuli (hyperalgesia), compared with the control Sprague-Dawley (SD) strain. The overarching hypothesis of this thesis is that alteration in the expression and/or functionality of the opioid system in key components of the descending pain pathway underpin hyperalgesia to inflammatory pain in the WKY rat, compared with SD counterparts.

The results show that WKY rats were hyporesponsive to the mu-opioid receptor (MOP) agonist morphine, administered systemically or into the ventrolateral periaqueductal grey (vlPAG), in the hot plate and formalin tests, compared to SD counterparts. Morphine-induced effects on neuronal activity in regions downstream of the vlPAG were blunted in WKY rats. WKY rats were also hyporesponsive to kappa-opioid receptor (KOP) modulators, U50488 and DIPPA, in response to acute heat and inflammatory stimuli. In addition, WKY rats displayed exaggerated mechanical allodynia compared to SD counterparts in the complete Freund's adjuvant (CFA) model of chronic inflammatory pain. Moreover, CFA induced a modest impairment in recognition memory in SD, but not in WKY, rats. Lastly, chronic SNC80 and DIPPA treatment had no effect on any of the behavioural domains (nociceptive responding/depression/cognition) in the CFA model in SD rats.

In conclusion, these findings suggest that the vlPAG is a key locus for hyporesponsivity to MOP agonism in the WKY rat and a deficit in MOP-induced recruitment of the descending inhibitory pain pathway may underlie hyperalgesia in the WKY rats. Alteration in the KOP system may also contribute to the WKY behavioural phenotype. Furthermore, the results also provide evidence for genotype-dependent exacerbation of nociceptive responding in the CFA model of chronic inflammatory pain. Taken together, these results extend our understanding on the role of the opioid system in altered nociceptive responding in the WKY rats genetically predisposed to negative affect.

Author's declaration

I hereby declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland Galway. The research is original and entirely my own with the following assistance:

Chapter 2

- Patricia Calcagno assisted during the behavioural tests.
- The gene expression data (except for PAG) were generated by Patricia Calcagno, and analysed by myself.
- Mass spectrometry and analysis of plasma morphine data were done by our industry collaborator Alkermes Inc in their facility.

Chapter 3

- Patricia Calcagno assisted during the behavioural tests.
- Western immunoblotting data were generated by Patricia Calcagno, and analysed by myself.

Chapter 4

- Patricia Calcagno assisted during the behavioural tests.
- The gene expression data (except for PAG) were generated by Patricia Calcagno and analysed by myself.

Chapter 5

- Patricia Calcagno assisted during the behavioural tests.

Chapter 6

- Dr Jessica Gaspar, Dr Sarah Jarrin, Patricia Calcagno, and Laura Boulton assisted during behavioural tests.

The thesis or any part thereof has not been submitted to the National University of Ireland or any other institution in connection with any other academic award.

Any views expressed herein are those of the author.

Signed: Mehrat Islam Fendoni

Date: Feb 26/2020

Acknowledgements

Firstly, I would like to thank my supervisor, Professor David Finn, for his guidance and mentorship, and more importantly for his patience, over the past four years. I would also like to thank my co-supervisors, Dr Michelle Roche and Professor John Kelly, for their persistent help and advice during these years. My sincere thanks to Dr Connie Sanchez, Dr Karen Smith, Dr David Eyerman, and Colleen Ellwanger from Alkermes Inc for their generous support and suggestions during the course of this project. I gratefully acknowledge Science Foundation Ireland and Alkermes Inc for funding my work through the Strategic Partnership Programme Grant (14/SPP/B3051).

To everyone in CNS, past and present, I honestly could not have worked with a better bunch of people than you. The camaraderie we share is absolutely unbeatable! You are my fabulous work family and my heartfelt thanks to each one of you for your time, help, and support.

To the Finns, you are the best! I have made friends for life here. Never ever was I alone when I needed one of you even just to rant and complain. We shared some good laughs and made memories that I surely will cherish forever. A special thank you to Dr Bright Okine for patiently guiding me through every little confusion I had in my first year. Jessica, thank you for keeping me sane!

To the Alks girls (or turtles as we once called ourselves), you are all fantastic! I will miss our shenanigans and the Taylor Swift playlist on our way to the meetings outside Galway.

Dr Daniel Kerr, you're my favourite Irish person. Words will not be enough to thank you for your help and support. Your enthusiasm is contagious! You always had a solution for my problems. Glad I could teach you (though with much difficulty) "shuprobhat" and "bagh".

Much appreciation goes to my Graduate Research Committee members for suggestions and advice regarding my work. Thank you to Ambrose and Silke and all the staff of the Pharmacology Department for your continuous support over the past few years.

I would also like to express my gratitude to Alan for his encouragement and mentorship. You have always been my guiding star!

A special shout-out to all my friends living in different time zones but being right by my side when I needed them. Last, but definitely not the least, I would like to thank my family who, despite being many thousands of miles away, has always been supportive of my choices. Thank you for believing in me and keeping me grounded. I would not have made this journey without you!

List of figures

List of tables

List of abbreviation

$5\text{-}HT_3$: 5-hydroxytryptamine type 3

5-HIAA: 5-hydroxyindoleacetic acid

3-CST: three-chamber sociability test

ACC: anterior cingulate cortex

Amyg: amygdala

ANCOVA: analysis of covariance

ANOVA: analysis of variance

AP: anteroposterior

Aq: aqueduct

ASIC: acid-sensing ion channel

BDNF: brain derived neurotropic factor

BLA: basolateral nucleus of amygdala

BSA: bovine serum albumin

Ca^{2+}: calcium

cAMP: cyclic adenosine monophosphate

CCI: chronic constriction injury

CCK: cholecystokinin

cDNA: complementary deoxyribonucleic acid

CeA: central nucleus of amygdala

CFA: complete Freund's adjuvant

CNS: central nervous system

COMT: catechol-O-methyltransferase

Cond: conditioning

CPA: conditioned place aversion

CPS: composite pain score

CRF: corticotropin-releasing factor

CSF: cerebrospinal fluid

Cyp: cyprodime

DHSC: dorsal horn of spinal cord

DMSO: dimethyl sulphoxide

DOP: delta-opioid receptor

dlPAG: dorsolateral periaqueductal grey

dmPAG: dorsomedial periaqueductal greys

DRG: dorsal root ganglia

DV: dorsoventral

ELISA: enzyme-linked immunosorbent assay

EPM: elevated plus maze

ERK: extracellular signal-regulated kinase

EZM: elevated zero maze

F-CPA: formalin-induced conditioned pain aversion

FST: forced swim test

GABA: gamma-aminobutyric acid

GFAP: glial fibrillary acidic protein

GPCR: G-protein-coupled receptor

HCl: hydrochloric acid

HB: hole board

HG: Hargreaves'

Hipp: hippocampus

HP: hot plate

HPA: hypothalamo-pituitary-adrenal

Hypo: hypothalamus

IASP: International Association for the Study of Pain

i.c.v.: intracerebroventricular

IHC: Immunohistochemistry

i.p.: intraperitoneal

i.pl.: intraplantar

i.t.: intrathecal

IQR: interquartile range

JNK: c-jun N-terminal kinase

K^+: potassium

KCC2: K^+-Cl^- co-transporter

KOP: kappa-opioid receptor

KW: Kruskal-Wallis

LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry

LDB: light/dark box

lPAG: lateral periaqueductal grey

LC: locus coeruleus

MAPK: mitogen activated protein kinases

MB: marble burying

MIA: monosodium iodoacetate

ML: mediolateral

MOP: mu-opioid receptor

Morph: morphine

MS: maternal separation

mPFC: medial PFC

mRNA: messenger ribonucleic acid

NIH: National Institute of Health

NMDA: N-methyl-D-aspartic acid

N/OFQ: nociceptin/orphanin FQ peptide

NOP: nociceptin/orphanin FQ receptor

NOR: novel object recognition

NRM: nucleus raphe magnus

NSAIDs: non-steroidal anti-inflammatory drugs

OB: olfactory bulbectomy

OD: optical density

OF: open field

OIH: opioid-induced hyperalgesia

PAG: periaqueductal grey

PB: phosphate buffer

PBN: parabrachial nucleus

PBS: phosphate buffered saline

PDYN: prodynorphin

PET: positron emission tomography

PENK: proenkephalin

PEAP: place escape/avoidance paradigm

PFA: paraformaldehyde

PFC: prefrontal cortex

PNOC: pronociceptin

p.o.: per oral

POMC: proopiomelanocortin

PPAR: peroxisome proliferator-activated receptor

PSNL: partial sciatic nerve ligation

PTSD: post-traumatic stress disorder

q.d.: once daily

RM ANOVA: repeated measures analysis of variance

RT-qPCR: real-time quantitative polymerase chain reaction

RVM: rostral ventromedial medulla

SART: specific alternation rhythm of temperature

s.c.: subcutaneous

SC: spinal cord

SD: Sprague-Dawley

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

SHR: spontaneously hypertensive rat

SIA: stress-induced analgesia

SIH: stress-induced hyperalgesia

SNC: sciatic nerve cuffing

SNI: spared nerve injury

SNK: Student-Newman-Keuls

SNL: spinal nerve ligation

SNP: single nucleotide polymorphisms

SPT: sucrose preference test

SRM: Selected Reaction Monitoring

T: time

TM: T-maze

TMJ: temporomandibular joint

TRP: transient receptor potential

TRPA1: transient receptor potential ankyrin 1

TRPV1: transient receptor potential vanilloid 1

TST: tail suspension test

Veh: vehicle

VF: von Frey

vlPAG: ventrolateral periaqueductal grey

WA: water avoidance

WKY: Wistar-Kyoto

List of publications and conference proceedings

Publications: Peer-reviewed journal articles

- *Review*

Ferdousi M and Finn DP (2018). Stress-induced modulation of pain: Role of the endogenous opioid system. Progress in Brain Research 239:121-177

- *Original research article*

Burke NN, **Ferdousi M**, Deaver DR, Finn DP, Roche M, Kelly JP (2019). Locomotor and anti-immobility effects of buprenorphine in combination with the opioid receptor modulator samidorphan in rats. Neuropharmacology 146:327-336

Ferdousi M, Calcagno P, Clarke M, Aggarwal S, Sanchez C, Smith, KL, Eyerman D, Kelly JP, Roche M, Finn DP. Hyporesponsivity to mu-opioid receptor agonism in the Wistar-Kyoto rat model of altered nociceptive responding associated with negative affective state. Pain (*manuscript under review*)

Conference proceedings/abstracts

- *Refereed or published abstracts/proceedings*

Ferdousi M, Calcagno P, Aggarwal S, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2019). Differential effect of kappa opioid receptor modulation in the Wistar-Kyoto rat model of hyperalgesia associated with negative affective state. *Irish Pain Society Annual Scientific Meeting*, Dublin, Ireland. October 2019. Poster presentation (Awarded 2nd Prize)

Calcagno P**, Ferdousi M**, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2019). Characterisation of behaviour and hippocampal opioid receptor expression in a chronic inflammatory pain model in Wistar-Kyoto versus Sprague-Dawley rats. *Irish Pain Society Annual Scientific Meeting*, Dublin, Ireland. October 2019. Poster presentation

Ferdousi M, Calcagno P, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2019). Characterisation of pain-, anxiety- and cognition-related behaviour in the complete Freund's adjuvant model of chronic inflammatory pain in Wistar-Kyoto rats. *32nd ECNP Congress*, Copenhagen, Denmark. September 2019. Poster presentation

McHugh K, Calcagno P, **Ferdousi M**, Sanchez C, Roche M, Finn DP, KellyJP (2019). Alterations in the central opioid system following acute swim stress and olfactory bulbectomy in the rat. *32nd ECNP Congress*, Copenhagen, Denmark. September 2019. Poster presentation

Ferdousi M, Calcagno P, Aggarwal S, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2019). Differential effect of kappa opioid receptor modulation in the Wistar-Kyoto rat model of hyperalgesia associated with negative affective state. *BNA 2019 Festival of Neuroscience*, Dublin, Ireland. Abstract PM172 (SP). April 2019. Poster presentation

Ferdousi M, Calcagno P, Clarke M, Aggarwal S, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2018). Hyporesponsivity to morphine in the Wistar-Kyoto rat model of hyperalgesia associated with negative affective state. *Annual Meeting of the Society for Neuroscience*, San Diego, USA. November 2018. Poster presentation

Ferdousi M, Calcagno P, Aggarwal S, Sanchez C, Kelly JP, Roche M, Finn DP (2018). Hyporesponsivity to morphine microinjected into the ventrolateral periaqueductal grey in Wistar-Kyoto versus Sprague-Dawley rats. *International Association for the Study of Pain (IASP) 17th World Congress on Pain*, Boston, USA. September 2018. Poster presentation

Ferdousi M, Calcagno P, Clarke M, Eyermann D, Sanchez C, Kelly JP, Roche M, Finn DP (2017). Evidence for Hyporesponsivity to Morphine in the Wistar-Kyoto Rat Model of Hyperalgesia Associated with Negative Affective State. *EFIC 2017*, Copenhagen, Denmark. September 2017. Poster presentation (Recognised as "Highly Recommended" poster)

Ferdousi M, Calcagno P, Clarke M, Eyermann D, Sanchez C, Kelly JP, Roche M, Finn DP (2017). Evidence for Hyporesponsivity to Morphine in the Wistar-Kyoto Rat Model of Hyperalgesia Associated with Negative Affective State. *Irish Pain Society Annual Scientific Meeting*, Galway, Ireland. August 2017. Poster presentation

Ferdousi M, Calcagno P, Clarke M, Eyermann D, Sanchez C, Kelly JP, Roche M, Finn DP (2017). Evidence for Hyporesponsivity to Morphine in the Wistar-Kyoto Rat Model of Hyperalgesia Associated with Negative Affective State. *Neuroscience Ireland Conference*, Galway, Ireland. August 2017. Oral and poster presentation

Ferdousi M, Calcagno P, Clarke M, Eyermann D, Sanchez C, Kelly JP, Roche M, Finn DP (2017). Evidence for Hyporesponsivity to Morphine in the Wistar-Kyoto Rat Model of Hyperalgesia Associated with Negative Affective State. *The Challenge of Chronic Pain Meeting 2017*, Cambridge, UK. Abstract: P25. March 2017. Poster presentation

Ferdousi M, Burke NN, Clarke M, Deaver DR, Roche M, Kelly JP, Finn DP (2016). Differential Expression of Opioid Receptor Genes in Brain Regions Involved in Pain and Negative Affect in Wistar-Kyoto versus Sprague-Dawley Rats. *Irish Pain Society Annual Scientific Meeting*, Dublin Ireland. September 2016. Poster presentation

- *Non-refereed/non-published abstracts/proceedings*

Ferdousi M, Calcagno P, Aggarwal S, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2019). Differential effect of kappa opioid receptor modulation in the Wistar-Kyoto rat model of hyperalgesia associated with negative affective state. *Galway Neuroscience Centre Annual Research Day*, NUI Galway, Ireland. December 2019. Poster presentation

Ferdousi M, Calcagno P, Aggarwal S, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2019). Differential effect of kappa opioid receptor modulation in the Wistar-Kyoto rat model of hyperalgesia associated with negative affective state. *College of Medicine, Nursing and Health Sciences Postgraduate Research Day*, NUI Galway, Ireland. May 2019. Oral presentation

Ferdousi M, Calcagno P, Aggarwal S, Sanchez C, Smith KL, Kelly JP, Roche M, Finn DP (2019). Differential effect of kappa opioid receptor modulation in the Wistar-Kyoto rat model of hyperalgesia associated with negative affective state. *Centre for Pain Research Annual Research Day*, NUI Galway, Ireland. March 2019. Oral presentation

Ferdousi M, Calcagno P, Clarke M, Aggarwal S, Madasu M, Sanchez C, Smith K, Eyerman D, Kelly JP, Roche M, Finn DP (2018). Hyporesponsivity to morphine in the Wistar-Kyoto rat model of hyperalgesia associated with negative affective state. *Galway Neuroscience Centre Annual Research Day*, NUI Galway, Ireland. December 2018. Poster presentation

Ferdousi M, Calcagno P, Aggarwal S, Sanchez C, Kelly JP, Roche M, Finn DP (2018). Hyporesponsivity to Morphine Microinjected into the Ventrolateral Periaqueductal Grey in Wistar-Kyoto versus Sprague-Dawley Rats. *College of Medicine, Nursing and Health Sciences Postgraduate Research Day*, NUI Galway, Ireland. May 2018. Oral presentation

Ferdousi M, Calcagno P, Clarke M, Eyermann D, Sanchez C, Kelly JP, Roche M, Finn DP (2017). Evidence for Hyporesponsivity to Morphine in the Wistar-Kyoto Rat Model of Hyperalgesia Associated with Negative Affective State. *Centre for Pain Research Annual Research Day*, NUI Galway, Ireland. March 2017. Poster presentation (Awarded 1st Prize)

Ferdousi M, Calcagno P, Clarke M, Eyermann D, Sanchez C, Kelly JP, Roche M, Finn DP (2017). Evidence for Hyporesponsivity to Morphine in the Wistar-Kyoto Rat Model of Hyperalgesia Associated with Negative Affective State. *College of Medicine, Nursing and Health Sciences Postgraduate Research Day*, NUI Galway, Ireland. May 2017. Poster presentation

Ferdousi M, Calcagno P, Clarke M, Eyermann D, Sanchez C, Kelly JP, Roche M, Finn DP (2016). Evidence for Hyporesponsivity to Morphine in the Wistar-Kyoto Rat Model of Hyperalgesia Associated with Negative Affective State. *Galway Neuroscience Centre Annual Research Day*, NUI Galway, Ireland. December 2016. Oral presentation

1. Chapter 1: General introduction

1.1. Pain

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP Task Force on Taxonomy, 1994). The experience of pain is complex and multidimensional. Pain can be considered to comprise of three aspects: sensory-discriminative (intensity, location, duration, temporal pattern, and quality of the stimulus), affective-motivational (associated unpleasantness and urge to escape the unpleasantness), and cognitive-evaluative (thoughts, beliefs, attitudes, intentions, and motivations related to pain). These dimensions of pain experience are processed in discrete but interconnected regions in the central nervous system (CNS) (Melzack and Casey, 1968; Price, 2000) and will be discussed in later sections.

The ability to perceive pain is required for survival and maintaining integrity of the organism. Depending on its duration, pain can be categorised as either acute or chronic. Acute pain, resulting from an intense noxious stimulus (e.g. after touching a hot surface), is adaptive in nature and protects the organism from injury or tissue damage. However, in the setting of persistent injury (and sometimes even in the absence of any noxious stimulus), components of the pain transmission pathway of both the peripheral and central nervous systems exhibit plasticity that enhances pain signalling, producing hypersensitivity. When these changes persist, it results in the maladaptive chronic pain condition (Basbaum et al., 2009).

Based on the aetiology, chronic pain (defined as pain persisting or recurring for more than 3 months) is inflammatory, neuropathic, or idiopathic in nature (Aguggia, 2003). It is generally characterised by exacerbated sensory symptoms, including allodynia (pain in response to normally non-painful/innocuous stimulus) and hyperalgesia (increased pain response to painful stimuli). Chronic pain has been associated with emotional distress, decrease in daily activities and occupational productivity, interference in social roles, thus affecting the overall quality of life of the patients (Breivik et al., 2006; Meints and Edwards, 2018). It is a significant global health problem and common in both developed (37%) and developing (41%) countries (Tsang et al., 2008). Pain also imposes a huge economic burden on society. The total healthcare cost associated with pain in the United

States has been estimated at $560 to $635 billion per year, with average annual cost of treating patients with chronic pain being three times higher than of individuals without chronic pain (Gaskin and Richard, 2012). In Ireland the total cost of treating chronic pain is estimated at €5.34 billion per year, with costs increasing according to the severity of pain (Raftery et al., 2012). Current approaches to pain management include pharmacological treatment with opioid analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), local anaesthetics, antidepressants, anticonvulsants, and gabapentinoids, as well as non-pharmacological methods such as cognitive-behavioural therapy, acupuncture, meditation, physiotherapy, medical devices, and psychotherapy (Coutaux, 2017; Hylands-White et al., 2017). However, the available treatment options are not always effective (Breivik et al., 2006) and associated with unwanted side effects such as nausea and vomiting, constipation, gastrointestinal irritation and ulceration, respiratory depression, and with some drugs, development of tolerance and addiction (Hylands-White et al., 2017).

Furthermore, the intensity of pain does not always correlate with total tissue damage following an injury or inflammation and can be influenced by emotionality. It is now widely accepted that stress and negative affective states (anxiety/depression) can exert a complex modulatory influence on pain experience (Asmundson and Katz, 2009; Wiech and Tracey, 2009; Jennings et al., 2014; Olango and Finn, 2014). In addition, individuals suffering from chronic pain have high rates of comorbid psychiatric disorders including anxiety and depression (Poole et al., 2009; Beesdo et al., 2010; Gadermann et al., 2012; de Heer et al., 2014), which may further reduce activity and quality of life of the patients. It has been reported that patients with chronic pain are more likely to present with depression (21% vs 10%) or anxiety (35% vs 18%) when compared to the general population (McWilliams et al., 2003, 2004). It has also been hypothesised that negative affect can influence the transition from acute to chronic pain (Linton, 2000). However, the causal association between pain and anxiety/depression is yet unclear, with findings supporting both an antecedent and consequent association (Knaster et al., 2012). Nevertheless, pain and negative affect share a complex reciprocal relationship, whereby one can aggravate the other, which support the concept of a self-perpetuating cycle of events that may underpin the chronic nature of such comorbid disorders. Therefore, a better understanding of the pain-negative affect interaction is warranted both from basic research and therapeutic standpoint.

1.2. Neural pathways mediating and modulating pain

A subpopulation of peripheral neurons called the nociceptors respond to intense and potentially noxious stimuli. These specialised neurons have their cell bodies in the dorsal root ganglia (DRG) for the body and the trigeminal ganglia for the face, head and neck region. The central axon of nociceptors innervates the dorsal horn of the spinal cord (DHSC) and the peripheral axon terminates in the target organ (soma or viscera). Based on the properties of axons, primary afferent neurons can be divided into three types: (1) large diameter myelinated Aβ-fibres of fast conduction velocity that respond to innocuous (non-nociceptive) mechanical stimulation (e.g. light touch, vibration); (2) medium diameter myelinated Aδ-fibres of intermediate conduction velocity that mediate acute, well-localised fast type of pain; and (3) thin unmyelinated C-fibres of slow conducting velocity that evoke a later poorly localised long-lasting dull pain (for review see Millan, 1999).

The peripheral endings of nociceptive primary afferents contain specialised transducer ion channels such as the transient receptor potential (TRP), acid-sensing ion channel (ASIC), and potassium channels (Woolf and Ma, 2007; Basbaum et al., 2009). Activation of these receptors in response to noxious stimuli of either thermal (heat or cold), mechanical, or chemical origin results in an influx of cations, generating action potential that relay nociceptive signal to the DHSC. In addition, a variety of substances (including glutamate, substance P, kinins, nitric oxide, histamine, adenosine, prostaglandins, and cytokines) released during tissue injury can directly activate or sensitise primary afferent neurons (Rosenthal, 1949; Stern et al., 1962; Hokfelt et al., 1975; Jahr and Jessell, 1983; Ferreira and Lorenzetti, 1994; Malmberg and Yaksh, 1994). The C-nociceptors project primarily to the superficial dorsal horn (laminae I and II) whereas the A-fibres terminate in the superficial layer (lamina I) as well as the deep layers (laminae III, IV, V, and X) of the dorsal horn (Willis, 1985). The primary afferent neurons synapse onto interneurons or second order neurons in the dorsal horn (or in the hindbrain in case of trigeminal ganglia). These second order neurons decussate (cross the midline to the contralateral side) at the spinal cord level and synapse onto the ascending fibres, relaying nociceptive signal to supraspinal (brain) regions (Nathan et al., 2001) (Figure 1.1).

1.2.1. Ascending pain pathways

The ascending pain projection pathways are highly complex and include the spinothalamic, spinoreticular, spinomesencephalic, spinoparabrachial, spinohypothalamic, and spinocervical tracts (Willis and Westlund, 1997; Millan, 1999; Almeida et al., 2004). Of these, the spinothalamic tract, which originates from the superficial and deep layers of the dorsal horn and projects to the thalamus and onward to the cerebral cortex, is particularly relevant to the sensory-discriminative and motivational-affective aspects of the pain experience (Hodge and Apkarian, 1990). The spinothalamic tract also projects collaterally to the midbrain periaqueductal grey (PAG) matter (Zhang et al., 1990). The spinothalamic tract can be further subdivided into three pathways: (a) the ventral spinothalamic tract, projecting to nuclei of the lateral complex of the thalamus and onto the somatosensory cortex, is involved in the sensory-discriminative component of pain; (b) the dorsal spinothalamic tract, projecting to the posterior medial and intralaminar nuclei of the thalamus, is involved in the emotional-cognitive aspects of pain; and (c) the monosynaptic spinothalamic pathway, projecting directly to the medial central nucleus of the thalamus, is also involved in the affective dimension of the pain experience (Albe-Fessard et al., 1985; Millan, 1999). Similar to the neurons of the spinothalamic pathway, the spinomesencephalic tract originates from the superficial and deep layers of the dorsal horn and projects to the midbrain including the PAG. This bundle is particularly important in mediating the autonomic and motivational-affective responses to pain (Willis and Westlund, 1997; Almeida et al., 2004).

The spinoreticular tract, which originates mainly from the deep layers and, to a lesser extent, superficial layer of the dorsal horn, carries nociceptive information to the brainstem and is more relevant to poorly localised pain (Basbaum et al., 2009). The afferents of the spinoreticular bundle predominantly innervate the medial reticular formations, including the rostral ventromedial medulla (RVM), and onward to the thalamus and hypothalamus. This tract is also involved in mediating the emotional aspect of pain but more importantly in the modulation of brainstem regions that are responsible for descending suppression (Lima et al., 1991; Almeida et al., 2004).

The spinoparabrachial tract originates from laminae I, II, and III of the dorsal horn and directly innervates the parabrachial nucleus (PBN) that, in turn, projects predominantly to the hypothalamus and amygdala and also to the PAG (Bernard et al., 1996; Gauriau and Bernard, 2002). In addition, the PBN receives indirect input from the spinoreticular

and spinomesencephalic fibres. The parabrachial pathway is involved in the thermal (heat and cold), mechanical, and visceral nociceptive processing along with autonomic, neuroendocrine, and motivational-affective responses to pain (Bester et al., 1997; Buritova et al., 1998; Millan, 1999).

Lastly, the neurons of the spinohypothalamic and spinocervical tracts originate mainly from the deep layers and, to a lesser extent, from the superficial lamina I of the dorsal horn. The spinohypothalamic fibres project to the hypothalamus (and onward to the thalamus, amygdala, and striatum) and are associated with the autonomic, motivational-affective, and alert responses to pain originating from the soma and viscera (Millan, 1999; Almeida et al., 2004). The spinocervical tract relays to the lateral cervical nucleus of the spine and onward to the thalamus that, in turn, projects further to the somatosensory cortices (Brown, 1981). The spinocervical bundle is involved in the sensory-discriminative, motivational-affective, and autonomic responses of pain (Millan, 1999).

Neuroimaging studies have demonstrated an extensive network of neural substrates, referred to as the "pain neuromatrix", that are activated by a noxious stimulus (Tracey and Mantyh, 2007). The thalamus is a key relay site for reception, integration, and transfer of nociceptive inputs to several cortical and limbic structures (Turner and Herkenham, 1991; Casey et al., 1994). Other areas including the PBN, PAG, prefrontal cortex (PFC), amygdala, hippocampus, striatum, and hypothalamus receive direct or indirect nociceptive inputs via ascending fibres (Derbyshire et al., 1997). Some of these regions are also involved in processing affect, emotion, motivation, and cognition. As such, these supraspinal regions accessed by multiple ascending pathways work in concert to produce sensory, affective, and cognitive aspects of pain experience. Moreover, these brain regions are themselves extensively interlinked and interact with mechanisms of descending modulation of pain at the level of DHSC as discussed below in section 1.2.2.

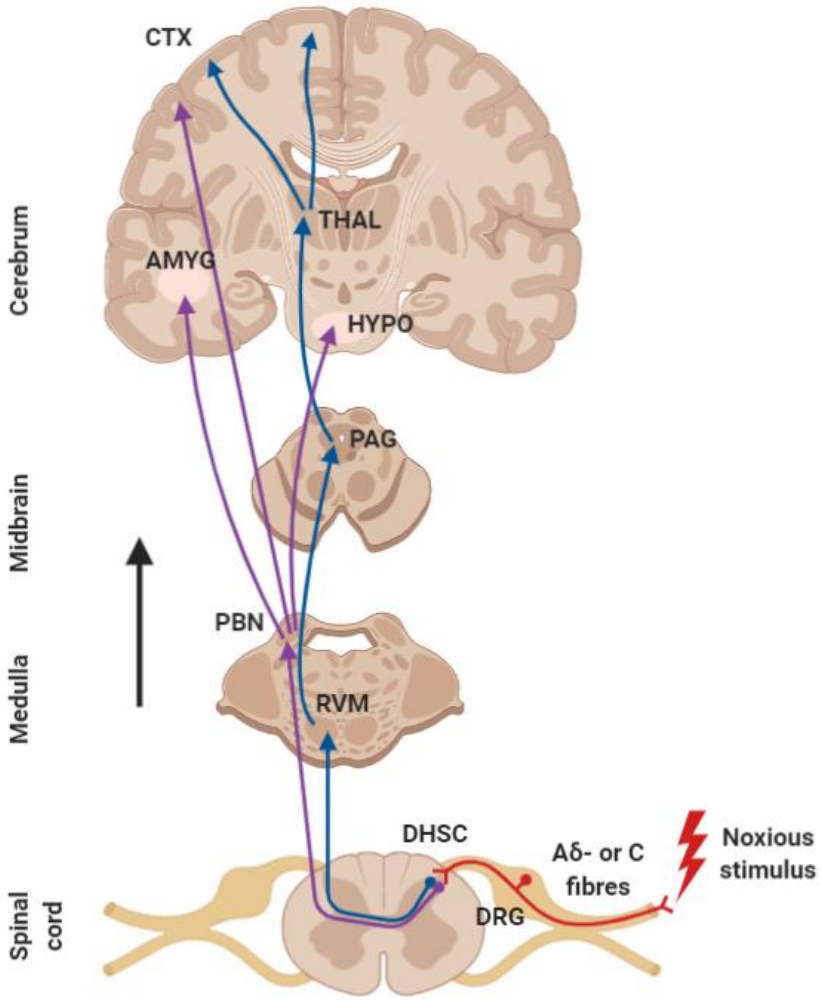


Figure 1.1: Ascending path pathways. Two of the main ascending pain pathways, spinothalamic and spinoparabrachial tracts, are depicted here (black arrow shows the direction of neurotransmission). Primary afferent (Aδ- or C-fibres, in red) neurons convey noxious information via the DRG to projection neurons within the DHSC. The spinothalamic pathway (in blue) transmits information to the cortex via the thalamus and provides information about the sensory-discriminative dimension of the painful stimulus. Neurons in the spinoparabrachial pathway (in purple) project to the PBN and onward to the hypothalamus, amygdala, and cortex, contributing to the affective-cognitive component of the pain experience. AMYG: amygdala, CTX: cortex, DRG: dorsal root ganglia, DHSC: dorsal horn of the spinal cord, HYPO: hypothalamus, PAG: periaqueductal grey, PBN: parabrachial nucleus, THAL: thalamus, RVM: rostral ventromedial medulla. Adapted from (Jennings, 2015).

1.2.2. Descending pain pathway

The gate-control theory, first described by Melzack and Wall in 1965, postulated the operation of a neural gate in the DHSC to control activation of ascending projections (Melzack and Wall, 1965). This theory, along with subsequent work involving chemical/electrical stimulation and pharmacological manipulation of specific supraspinal regions (Reynolds, 1969; Mayer et al., 1971; Yeung et al., 1977; Willis, 1988; Fields et al., 1991), has greatly contributed to the understanding of the central mechanisms that modulate pain. The descending pain modulatory pathway is now a well-characterised anatomical network that enables regulation of nociceptive processing under various circumstances to either supress (inhibition) or enhance (facilitation) nociception (for review see Millan, 2002) (Figure 1.2). Descending pathways, originating from cerebral structures, modulate nociception generally by altering the release of neurotransmitters [e.g. noradrenaline, serotonin, dopamine, endogenous opioids, gamma aminobutyric acid (GABA)] from neurons largely within the DHSC that include the terminals of primary afferent neurons, secondary sensory neurons, and interneurons (Basbaum and Fields, 1984; Millan, 1999). Interestingly, the substrates and supraspinal regions involved in descending inhibition and facilitation of nociception are often identical (Millan, 2002).

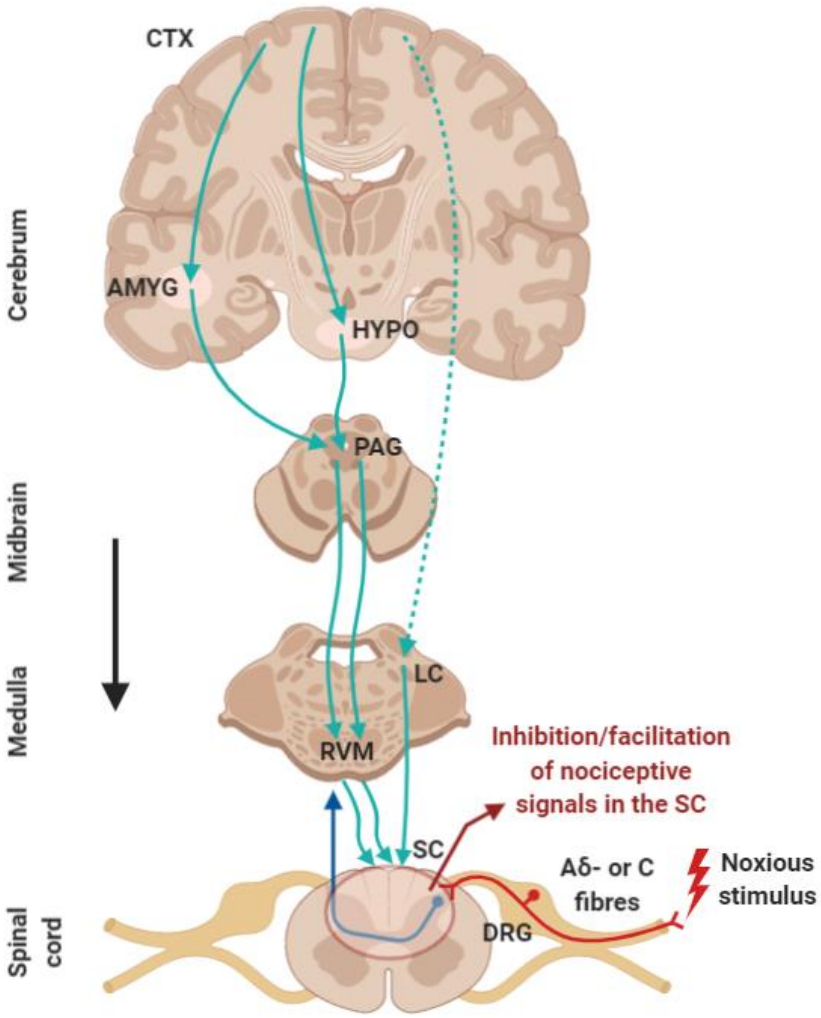


Figure 1.2: Descending pain modulatory pathway. Neurons (in cyan) originating from higher brain regions such as the cortex, amygdala, and hypothalamus project to the PAG and RVM and finally to the SC where modulation (inhibition or facilitation) of nociceptive transmission occurs (black arrow shows direction of neurotransmission). Cortical areas can also project to the LC (dotted cyan line). The LC, in turn, projects directly to the dorsal horn and modulate nociceptive neurotransmission in the SC. AMYG: amygdala, CTX: cortex, DRG: dorsal root ganglia, HYPO: hypothalamus, LC: locus coeruleus, PAG: periaqueductal grey, RVM: rostral ventromedial medulla, SC: spinal cord. Adapted from (Jennings, 2015).

1.2.2.1. Descending inhibition

Descending inhibition involves suppression of the activity of ascending pain pathways by supraspinal regions, which include the hypothalamus, locus coeruleus (LC), PBN, RVM, PAG, and limbic (amygdala) and cortical (insula, cingulate, frontal and parietal) areas (Millan, 2002). These higher brain regions with projections descending directly to the dorsal horn, primarily the superficial layers (Basbaum et al., 1978; Ruda et al., 1981), modulate nociceptive neurotransmission at the level of the spinal cord (Willis and Westlund, 1997; Millan, 2002). The basolateral nucleus of amygdala (BLA) receives highly processed information from the thalamus and cortex (prefrontal, insular, and anterior cingulate cortices) (McDonald, 1987; Augustine, 1996; Shi and Davis, 1999) and projects onto the central nucleus of amygdala (CeA) (Paré et al., 1995; Pitkänen et al., 1997). The CeA serves as a major output toward the hypothalamus (Hopkins and Holstege, 1978; Bourgeais et al., 2001) and brainstem regions including the PAG (Hopkins and Holstege, 1978; Da Costa Gomez and Behbehani, 1995a; Pitkänen et al., 1997; Oka et al., 2008). The PAG is also interconnected with the PBN (Mantyh, 1983; Gauriau and Bernard, 2002) that, in turn, projects onto the spinal cord (Ma and Peschanski, 1988; Yoshida et al., 1997). Additionally, the PAG receives afferents from the hypothalamus, and limbic forebrain regions (Keay and Bandler, 2001). Finally, the PAG relays to the RVM that further projects to the spinal dorsal horn (Basbaum and Fields, 1979; Beitz et al., 1983; Mantyh, 1983; Fields et al., 1995). The RVM can modulate nociceptive neurotransmission at the level of spinal cord through the activity of its ON and OFF cells, with OFF cells suppressing pain (descending inhibition) (Fields et al., 1977, 1983a; Heinricher et al., 1989; Millan, 2002). Hence, the amygdala, PAG, and RVM comprise major sites for this “top-down” pain modulating system which, when activated, either from within the PAG, RVM or higher centres, elicits antinociceptive response by inhibiting nociceptive transmission at the DHSC (Millan, 2002).

1.2.2.2. Descending facilitation

Descending facilitation refers to the potentiation of ascending transmission of nociceptive information from the DHSC via activity of top-down pathways (Porreca et al., 2002; Zhuo, 2017). Under basal conditions, the majority of nociceptive dorsal horn neurons are under tonic descending influence. Hence, a ‘balance’ between facilitation and inhibition dampens excessive sensitivity to a noxious stimulus, maintaining a baseline state of sensory processing that can be modified depending on physiological demand (Laird and

Cervero, 1990; Watkins et al., 1994). However, under pathological conditions, persistent nociceptive input can induce neuroplastic changes that perturb this balance, favouring a sustained facilitatory influence that manifests behaviourally as enhanced pain (Vanegas and Schaible, 2004).

Descending facilitation has been proposed to contribute to chronic pain states and subsequent development and maintenance of hyperalgesia and/or allodynia (Urban et al., 1996; Pertovaara, 1998; Wang et al., 2013a) and possibly pain-associated emotional disorder (Zhuo, 2016). Furthermore, descending facilitatory influences have been implicated in paradoxical increased pain conditions caused by sustained opioid exposure (Vanderah et al., 2001). The neurobiological mechanisms underlying sustained facilitation of nociceptive information that result in a chronic pain state remain poorly understood. However, it has been suggested that increased nociceptive stimuli due to tissue injury or inflammation elicit enhanced afferent input to the spinal dorsal horn (Porreca et al., 2002). Such sustained afferent input has been hypothesised to initiate a facilitated pain state (Baron et al., 2013) since application of a local anaesthetic (lidocaine) to the site of nerve injury prevents development of tactile and thermal hypersensitivity (Malan et al., 2000).

Anatomically, there is no separation between the supraspinal regions involved in descending inhibition and facilitation (Millan, 2002). Of particular importance is the RVM that can either facilitate or inhibit spinal nociceptive transmission via activation of ON and OFF cells, respectively (Heinricher et al., 1989). Converging evidence suggests that an increase in ongoing activity of RVM ON cells may contribute to the descending facilitation and consequently exaggerated pain response (Bederson et al., 1990; Neubert et al., 2004; Kincaid et al., 2006; Sanoja et al., 2010; Cleary and Heinricher, 2013). Although most studies examining descending facilitation have focused on the RVM-spinal cord connection, higher brain regions may also influence this phenomenon. One such emerging region of interest is the anterior cingulate cortex (ACC). Several studies have reported that activation of ACC enhances nociceptive transmission directly in spinal dorsal horn (Chen et al., 2014; Kang et al., 2015) or indirectly in downstream brain regions, including the RVM (Calejesan et al., 2000), which then facilitates spinal nociception. Taken together, these studies suggest contribution of both cortical and subcortical regions in the top-down descending facilitation of pain.

1.2.3. Pathophysiology of chronic pain

Normally descending pain facilitatory and inhibitory systems function in concert, maintaining a baseline state of sensory processing (Vanegas and Schaible, 2004). This equilibrium can be modified during an acute injury or inflammation that generally resolves, restoring the balance between descending inhibition and facilitation. However, for reasons that are not yet understood completely, an imbalance in descending pain modulation can persist over time even after the removal of the causal stimulus, resulting in pathological chronic pain states (Terayama et al., 2000; Porreca et al., 2002; Kwon et al., 2014; Ossipov et al., 2014). The mechanism of chronic pain is complex and growing evidence indicates that the development and maintenance of chronic pain state involves alteration in pain modulatory pathways and hypersensitisation of the peripheral and central nervous systems (Aguggia, 2003; Basbaum et al., 2009).

Persistent pain associated with injury or diseases (e.g. arthritis) can result from alterations in the properties of peripheral nerves, leading to increased spontaneous firing and decreased activation threshold (Aguggia, 2003; Basbaum et al., 2009). Peripheral sensitisation commonly occurs as a consequence of tissue damage that results in the accumulation of inflammatory agents (such as substance P, prostaglandins, bradykinin, neurotrophins, cytokines, and chemokines) within or near the site of injury. These endogenous factors, collectively referred to as the "inflammatory soup", reduce threshold of nociceptors, thereby enhancing excitability of primary afferent fibres and heightening their sensitivity to noxious (hyperalgesia) or innocuous (allodynia) stimuli (Curatolo et al., 2006). This phenomenon results in an amplified nociceptive input to the spinal cord, which in turn may cause an increase in neuronal activity in the dorsal horn, known as central sensitisation.

Central sensitisation refers to a state of hyperexcitability in the CNS, leading to enhanced processing of nociceptive signals (Woolf, 1983). Abnormal sensory processing, which persists beyond the normally expected timecourse of tissue insult, can induce neuroplastic changes within descending pain pathways, perturbing the balance between inhibitory and facilitatory activities (Porreca et al., 2002; Zhuo, 2017). Hence, increased activity of the descending facilitatory pain pathway may contribute to central sensitisation that, in turn, can lead to the development of chronic pain. Moreover, reduced top-down recruitment of descending inhibition has been implicated in chronic pain (Terayama et al., 2000; De Felice et al., 2011; Cheriyan and Sheets, 2018). Other mechanisms, such as increased

glutamatergic neurotransmission and reduced GABAergic interneuron activity in spinal dorsal horn, alteration in serotonergic neurotransmission in different brain sites, and activation of microglia and astrocytes in the spinal cord, may contribute to enhanced facilitation and diminished inhibition of nociceptive signals, resulting in manifestation of chronic pain (Zhang et al., 2000; Rahman et al., 2006; Torsney and MacDermott, 2006; Keller et al., 2007; Basbaum et al., 2009; Beggs and Salter, 2013).

1.3. Emotional modulation of pain

1.3.1. Interactions between pain and stress/affect

Stress is experienced by all animals and generally refers to a biological response to a potentially dangerous or threatening situation. Stress causes a disturbance in the homeostasis of an organism which leads to alterations in physiology and behaviour (Chrousos, 2009). Stress can exert a complex bidirectional effect on pain. Depending on the nature, intensity, and duration of the stressful or aversive stimulus, nociceptive responding is either reduced (stress-induced analgesia, SIA) or exacerbated (stress-induced hyperalgesia, SIH) during and/or following exposure to stress. SIA is a potent form of pain suppression, typically occurring during or following a stressful stimulus that is robust and intense but acute in nature. SIA is an innate adaptive response and therefore has protective, survival value (Bolles and Fanselow, 1980). A plethora of studies has demonstrated conditioned and unconditioned SIA in humans and rodents and mechanisms underlying this phenomenon have been studied extensively in animal models (Yamada and Nabeshima, 1995; Ford and Finn, 2008; Butler and Finn, 2009). A greater understanding of the neurobiological mechanisms underpinning SIA will aid in understanding of endogenous analgesia and inform the development of novel therapeutic strategies to better treat pain.

On the other hand, stress can also heighten sensitivity to, or exacerbate the experience of, pain via the phenomenon of SIH. Prolonged or repeated exposure to physical or psychological stress may result in maladaptive alterations, resulting in exacerbated nociceptive responding in humans (Rhudy and Meagher, 2000; Dufton et al., 2008; Kuehl et al., 2010; Crettaz et al., 2013; Ortego et al., 2016) and rodents (Imbe et al., 2004; Gameiro et al., 2006; Chung et al., 2009; Dina et al., 2009; Le Roy et al., 2011; Quintero et al., 2011; Qi et al., 2016). For instance, stress can amplify intensity and severity of perceived pain in irritable bowel syndrome (Blanchard et al., 2008; Popa et al., 2018),

headaches (Schramm et al., 2015), and abdominal pain (Walker et al., 2012). In addition, it is now well-documented that stress can amplify existing symptoms associated with chronic pain. Thus, stress is an important aetiological factor for a number of chronic pain disorders such as musculoskeletal pain disorders (including arthritis and neck/low back pain), complex regional pain syndrome, and fibromyalgia (Grande et al., 2004; Nilsen et al., 2007; Zautra et al., 2007; Kraatz et al., 2013; Heidari et al., 2016; Buscemi et al., 2019). Although SIH is of high clinical relevance, neurobiological mechanisms underlying this phenomenon are relatively less studied.

Stress can also be a predisposing factor for neuropsychiatric disorders, including anxiety and major depressive disorder (Davidson and McEwen, 2012). Moreover, negative affective states (anxiety and depression) share a complex reciprocal relationship with pain, and this also constitutes a particular area of clinical interest within the context of stress-pain interactions. Alteration (an exacerbation in most cases) in nociceptive responding has been reported in patients with anxiety and depression (Bair et al., 2008, 2013; Kroenke et al., 2011), as well as in rodent models of negative affect (Zeng et al., 2008; Burke et al., 2010, 2013b; Rivat et al., 2010; Shi et al., 2010; Zhang et al., 2012; Ide et al., 2015). The relationship between chronic pain and altered emotional states is complex and it is difficult to ascertain which condition precedes or predisposes the other. There is considerable overlap in the neurobiological pathways (including the descending modulatory pain pathway) and substrates implicated in both pain and stress/affect. Thus, it is postulated that these closely linked neural systems can modulate one another reciprocally and any maladaptive change in one system may have consequences in another, leading to debilitating comorbid conditions. The following sections will give brief overviews of the comorbidity between pain and negative affect in the clinical population, the preclinical models used to investigate SIH and pain-negative affect interaction (with particular emphasis on the Wistar-Kyoto rat model), and the common supraspinal and spinal neural substrates implicated in SIH.

1.3.2. Comorbidity between pain and anxiety and depression

Chronic pain is a major unmet health problem of current times, causing emotional distress and impairing quality of life, work, and functioning in daily life. Epidemiological studies have indicated that chronic pain is one of the leading causes of global non-fatal health loss measured in terms of years lost to disability (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). The prevalence of chronic pain of moderate to

severe intensity is 19% in Europe alone (Breivik et al., 2006, 2013; Reid et al., 2011). In Ireland, this prevalence is 35.5% and the total healthcare cost associated with chronic pain has been estimated to be €5.34 billion per year, or annually €5,665 per patient (Raftery et al., 2011, 2012; Gannon et al., 2013).

In addition, a growing body of literature indicates that chronic pain and psychiatric disorders such as anxiety and depression are highly comorbid (Velly and Mohit, 2018). Clinical studies have revealed a high prevalence of chronic pain disorders and altered pain thresholds and tolerance in patients with anxiety or depression (Lautenbacher et al., 1999; Bair et al., 2003, 2008; Asmundson and Katz, 2009). Patients with anxiety or depression are more likely to report pain complaints, both in terms of frequency and severity, compared to their healthy cohort (Pompili et al., 2012; de Heer et al., 2014). Moreover, occurrence of depressive symptoms could predict future disposition to various musculoskeletal pain conditions such as low back and neck/shoulder pain (Ohayon and Schatzberg, 2003). Conversely, patients with chronic pain are more likely to present with depression or anxiety than individuals without pain (McWilliams et al., 2003, 2004; Beesdo et al., 2010; de Heer et al., 2014; Taloyan and Lofvander, 2014). Other reports have stated that the prevalence of anxiety or depression in patients with chronic pain may be as high as 50% or 80%, respectively (Poole et al., 2009; Gadermann et al., 2012). Thus, comorbid chronic pain and stress-related affective disorders further aggravate the severity of each individual disorder, resulting in poor therapeutic outcome and quality of life for patients and imposing a huge socioeconomic burden.

1.3.3. Preclinical models of hyperalgesia associated with stress and negative affective state

As mentioned earlier, the underlying neurobiological and molecular mechanisms of interactions between stress/negative affect and pain are poorly understood. As such, there has been growing interest in elucidating the mechanistic underpinnings of hyperalgesia co-occurring with stress and/or anxiety and depression, which might then be harnessed to develop more effective therapeutic strategies. In this respect, preclinical studies reveal critical information for the understanding of the neurobiological mechanisms underlying these comorbid conditions and their dynamic interactions, and also provide animal models with which to assess putative novel treatments. A relatively large number of well-characterised animal models have been employed to study SIH that generally involve repeated or persistent application of a psychological and/or physical stressor (e.g. forced

swim stress, restraint stress, social defeat, maternal separation/deprivation, chronic unpredictable mild stress) for several days to weeks, in combination with a test for assessing nociceptive responding (spontaneous or evoked). The descriptions of these SIH models and the effects of different stressor paradigms on nociceptive responding in rodents have been reviewed in great detail by Jennings and coworkers (Jennings et al., 2014) and will be discussed in section 1.4.6 of this chapter in the context of the role of opioid system in mediating SIH. Furthermore, in the past several years, emerging research has also examined the relationship between pain and negative affect (anxiety and depression) in animal models. In general, these studies can be categorised into three groups (see Table 1.1 and 1.2): (1) studies that examined whether pain leads to anxiety-/depression-like behaviours; (2) studies that examined behavioural manipulations precipitating anxiety and depression-like behaviours alter nociceptive responses; and (3) studies that simultaneously examined nociceptive responses and negative affect. On the whole, these studies provide strong evidence for the complex bidirectional interaction between pain and negative affect with persistent/chronic pain aggravating emotional disorders whilst negative affective states exacerbating nociceptive responses. It should also be noted that some discrepancies are observed in the behavioural outcomes. In our view, these inconsistencies might be due to, but not limited to, use of different animal species, behavioural assays, pain modalities, time points for behavioural tests, and other subtle experimental variables.

Table 1.1: Preclinical studies examining whether chronic inflammatory or neuropathic pain leads to anxiety-/depressive-like behaviours

Model	Species/ strain	Behavioural test(s)	Key behavioural findings	Reference
Studies examining if pain induces and/or exacerbates anxiety				
Carrageenan	SD rats	EZM	Anxiety-like behaviour (5-6 h)	(Ji et al., 2007)
CFA	C57Bl/6J mice	EPM, LDB	Anxiety-like behaviour (week 4)	(Narita et al., 2006a)
	C57Bl/6J mice	OF, EPM	Anxiety-like behaviour (week 1)	(Chen et al., 2013; Yue et al., 2018)
	C57Bl/6J mice	OF, EPM	Anxiety-like behaviour (week 3)	(Wang et al., 2015a; Guo et al., 2016; Sun et al., 2016b)
	C57Bl/6J mice	OF	No anxiety-like behaviour (Day 2 and 5)	(Sheahan et al., 2017)
	Balb/c and C57BL/6 mice	OF, EZM, MB	No anxiety-like behaviour (week 4)	(Urban et al., 2011)
	C57Bl/6J mice	OF, EPM	No anxiety-like behaviour (10 days)	(Liu et al., 2015)
	SD rats	OF, EPM, LDB	Anxiety-like behaviour (week 4)	(Parent et al., 2012)
	SD rats	EZM	Anxiety-like behaviour (week 4)	(Wu et al., 2017)
	Wistar rats	EPM, LDB	Anxiety-like behaviour (Day 1, 3, and 10)	(do Nascimento and Leite-Panissi, 2014)
SNL	SD rats	OF, EPM, LDB	No anxiety-like behaviour (week 2)	(Kontinen et al., 1999)
	SD rats	OF, EPM	Anxiety-like behaviour (week 2)	(Jiang et al., 2014a)
SNI	Wistar and Wistar Han rats	OF, EPM	Anxiety-like behaviour (week 3-4)	(Leite-Almeida et al., 2012; Avila-Martin et al., 2015)
	Long–Evans rats	OF, EPM	Anxiety-like behaviour (week 20-24)	(Seminowicz et al., 2009; Low et al., 2012)
	Balb/c and C57BL/6 mice	OF, EZM, MB	No anxiety-like behaviour (week 4)	(Urban et al., 2011)
	C57BL/6 mice	EPM, HB, LDB	Anxiety-like behaviour (week 4-6)	(Sieberg et al., 2018)
	C57BL/6 mice	Emergence, novelty suppressed feeding	Anxiety-like behaviour (week 2)	(Mutso et al., 2012)
CCI	Wistar rats	EPM	Anxiety-like behaviour (week 3-4)	(Roeska et al., 2008)

	Balb/c and C57BL/6 mice	OF, EZM, MB	No anxiety-like behaviour (week 4)	(Urban et al., 2011)
SNC	C57Bl/6J mice	EPM, MB	Anxiety-like behaviour (week 4-5)	(Benbouzid et al., 2008)
PSNL	C57Bl/6J mice	EPM, LDB	Anxiety-like behaviour (week 4)	(Narita et al., 2006a, 2006b; Matsuzawa-Yanagida et al., 2008; Sawada et al., 2014)
	ddY mice	OF	No anxiety-like behaviour (week 1)	(Kodama et al., 2011)
Studies examining if pain induces and/or exacerbates depression				
CFA	Wistar rats	FST	Depressive-like behaviour (week 1-2)	(Kim et al., 2012)
	Swiss mice	FST, TST	Depressive-like behaviour (week 1-3)	(Maciel et al., 2013)
SNL	SD rats		Depressive-like behaviour (week 4)	(Hu et al., 2010)
SNI	C57BL/6 mice	FST	Depressive-like behaviour (week 1)	(Norman et al., 2010)
	SD rats	FST, SPT	Depressive-like behaviour (week 2)	(Wang et al., 2011; Goffer et al., 2013)
CCI	Wistar rat	FST	Depressive-like behaviour (week 3-4)	(Hu et al., 2009)
	SD rats	FST	Depressive-like behaviour (week 2-3)	(Fukuhara et al., 2012)
	ICR mice	FST, TST	Depressive-like behaviour (week 2-6)	(Zhao et al., 2014)
	C57Bl/6J mice	SPT	Depressive-like behaviour (week 4-11)	(Dellarole et al., 2014)
	Swiss mice	FST	Depressive-like behaviour (week 2)	(Jesse et al., 2010)
PSNL	C57Bl/6J mice	SPT	Depressive-like behaviour (week 1-2)	(Bura et al., 2013)
	Swiss mice	FST, TST	Depressive-like behaviour (week 4)	(Gai et al., 2014)
Studies examining if pain induces and/or exacerbates both anxiety and depression				
Kaolin/ carrageenan	Wistar rats	EPM, FST, SPT	Anxiety- and depressive-like behaviour (week 4)	(Amorim et al., 2014)
CFA	SD rats	MB, EZM, FST	Anxiety- and depressive-like behaviour (week 4)	(Borges et al., 2014)
	SD rats	OF, SPT	Anxiety- and depressive-like behaviour (week 3-4)	(Grégoire et al., 2014)
	SD rats	OF, EPM, FST, SPT	Anxiety-like behaviour (week 3-4) but no depressive-like behaviour	(Zhong et al., 2019)
	C57BL/6 mice	EZM, SPT	Anxiety- and depressive-like behaviour (Day 1-2)	(Refsgaard et al., 2016)

	C57BL/6N or C57BL/6J mice	OF, EPM, LDB, HB, FST	No anxiety- or depressive-like behaviour (week 2)	(Pitzer et al., 2019)
SNL	C57BL/6 mice	OF, EPM, LDB, FST	Anxiety- (week 4) and depressive-like behaviour (week 2-4)	(Suzuki et al., 2007)
	SD rats	EPM, SPT	Anxiety- and depressive-like behaviour (week 4)	(Ji et al., 2017)
SNI	C57BL/6 mice	OF, EPM, FST, SPT	Anxiety- and depressive-like behaviour (week 8-9)	(Descalzi et al., 2017)
	C57BL/6N or C57BL/6J mice	OF, EPM, LDB, HB, FST	No anxiety- or depressive-like behaviour (week 12)	(Pitzer et al., 2019)
	Wistar-Han rats	OF, EPM, FST	Anxiety- (week 4) and depressive-like behaviour (week 4)	(Leite-Almeida et al., 2009)
	Wistar-Han rats	OF, EPM, FST	Increased immobility but no anxiety-like behaviour (7 weeks)	(Gonçalves et al., 2008)
	SD rats	EPM, FST, SPT	Anxiety- and depressive-like behaviour (week 4)	(Satyanarayanan et al., 2019)
CCI	Wistar-Han rats	EPM, FST	Anxiety- (week 3) and depressive-like behaviour (week 5)	(Caspani et al., 2014)
	SD rats	EPM, FST	Anxiety- and depressive-like behaviour (week 1-3)	(Li et al., 2014)
	SD rats	OF, EPM, SPT	Anxiety-like behaviour (week 2-3) but no decreased sucrose preference	(Gregoire et al., 2012)
	SD rats	EZM, FST	Anxiety- and depressive-like behaviour (week 4)	(Alba-Delgado et al., 2013)
SNC	C57BL/6 mice	LDB, MB, novelty suppressed feeding, FST, splash	Anxiety- (week 4-8) and depressive-like behaviour (week 6-8)	(Yalcin et al., 2011)
	C57BL/6 mice	OF, EZM, FST	Anxiety- (week 5) and depressive-like behaviour (week 6)	(Dimitrov et al., 2014)
PSNL	C57Bl/6J mice	OF, EPM, TST	No anxiety- or depressive-like behaviour (week 1-4)	(Hasnie et al., 2007)
	SD rats	OF, EPM, FST, SPT	Anxiety-like behaviour (week 4) but no depressive-like behaviour	(Wang et al., 2015d)

Table 1.2: Preclinical studies examining whether negative affect induces and/or exacerbates nociceptive response.

Model of negative affect	Species/ strain	Behavioural test(s)	Key behavioural findings	Reference
Genetic (anxiety and depression)	WKY rats	Hot plate, tail flick	Increased sensitivity on the hot plate but tail flick test	(Burke et al., 2010)
	WKY rats	Formalin	Increased sensitivity	(Burke et al., 2010; Rea et al., 2014)
	WKY rats	von Frey	No difference	(Burke et al., 2010)
	WKY rats	von Frey	Increased sensitivity	(Taylor et al., 2001; Hestehave et al., 2019a)
	WKY rats	CCI: von Frey	Increased allodynia	(Zeng et al., 2008)
	WKY rats	TMJ: algometer	Increased allodynia	(Wang et al., 2012)
	WKY rats	MIA: von Frey	Increased allodynia	(Burston et al., 2019)
OB (depression)	Wistar rats	Paw pressure	Decreased sensitivity	(Belcheva et al., 2009)
	SD rats	Hargreaves'	Decreased sensitivity	(Su et al., 2010)
	SD rats	Hargreaves'	Decreased sensitivity	(Wang et al., 2010; Jiang et al., 2014b)
		Formalin	Increased sensitivity	
	Lister-Hooded rats	von Frey, formalin	Increased sensitivity	(Burke et al., 2010)
		Hot plate, tail flick	No difference	
	SD rats	SNL: von Frey, acetone	Increased allodynia	(Burke et al., 2013a, 2014)
		SNL: Hargreaves'	No difference	
	SD rats	SNL: von Frey, Hargreaves'	Decreased sensitivity	(Wei et al., 2017)

1.3.3.1. Wistar-Kyoto rat: a genetic model of hyperalgesia associated with negative affect

When investigating the influence of negative affect on nociceptive responding, one important aspect to consider is genetics. In this regard, the inbred Wistar-Kyoto (WKY) rat strain is a genetic model of negative affect and exhibits hypersensitivity to both stress and pain. Originally, the WKY strain was developed from the Wistar rat to serve as the normotensive control strain for the spontaneously hypertensive rats (SHR) (Okamoto and Aoki, 1963). However, it was later noted that the behaviour of the WKY rats was not 'normal', resulting in a series of investigations that established the WKY rat strain as a genetic model of stress hyperresponsivity, anxiety and depression. As an inbred strain, the WKY rats offer the advantage to dissociate genetic and environmental components of a trait since the animals, in theory, are identical to each other genotypically. Hence, the WKY rat is a useful model for studying the neurobiology of anxiety and depression, and their tendency to alter nociceptive responding.

WKY rats display aberrant behavioural and neurochemical responses to stress compared to commonly used outbred rat strains. WKY rats are more prone to develop stress-induced gastric ulcers (Paré, 1989c, 1989a; Paré and Redei, 1993). Physiologically, these rats have altered neuroendocrine stress responses, including exaggerated hypothalamic-pituitary-adrenal (HPA) axis responses to stress (Redei et al., 1994; Rittenhouse et al., 2002; De La Garza and Mahoney, 2004) and sustained elevated levels of corticosterone and adrenocorticotropic hormone in plasma (Bucher et al., 1987; Paré and Redei, 1993; Pardon et al., 2002; Malkesman et al., 2006). Moreover, turnover of monoamines (serotonin, noradrenaline, and dopamine) and their metabolites is altered in the PFC, nucleus accumbens and hippocampus, regions implicated in anxiety and depression, in the WKY rats compared with outbred Sprague-Dawley (SD) and Wistar rats (Pardon et al., 2002; De La Garza and Mahoney, 2004; Ferguson and Cada, 2004; Scholl et al., 2010; Shetty and Sadananda, 2017).

The anxiety and depression-like phenotype of the WKY rats have been reported by several groups. Compared to other rat strains, the WKY rats exhibit increased immobility in the forced swim test (Paré, 1992; Paré and Redei, 1993; Tejani-Butt et al., 1994, 2003; Lopez-Rubalcava and Lucki, 2000; Rittenhouse et al., 2002; Burke et al., 2010; Nam et al., 2014), indicating behavioural despair which is linked to depression-like behaviour in rodents (Slattery and Cryan, 2012). WKY rats show anhedonia in the sucrose preference

test (Malkesman et al., 2005; Burke et al., 2016; D'Souza and Sadananda, 2017), which is again indicative of depressive-like phenotype. Of interest, noradrenergic and dopaminergic, but not serotonergic, modulating antidepressant drugs can reverse the increased immobility displayed by WKY rats in the forced swim test (Lahmame et al., 1997; Lopez-Rubalcava and Lucki, 2000; Tejani-Butt et al., 2003). In addition, WKY rats spend reduced time in the aversive zones of the open field and elevated plus maze (Gentsch et al., 1987; Paré, 1989b, 1994; Tejani-Butt et al., 1994; Malkesman et al., 2005; Malkesman and Weller, 2009; Burke et al., 2010, 2016), indicating anxiety-like behaviour. Moreover, WKY rats display anxiety-vulnerable behavioural inhibition trait in a variety of situations (McAuley et al., 2009), such as increased immobility and higher latency to leave the centre of the open field (Paré, 1989c; Berton et al., 1997; O'Malley et al., 2010), increased freezing in a novel environment (Nosek et al., 2008), and increased time spent in the centre zone of the elevated plus maze arena (Nam et al., 2014). Although WKY rats exhibit pronounced hypolocomotor activity in the open field (Paré, 1989a; Pardon et al., 2002; Burke et al., 2010), deficit in motor activity is not seen in home cage activity (Burke et al., 2016) or in the rotarod test (Ferguson et al., 2003). It has been suggested that the WKY rats may not have an overt deficit in motor ability, but their deficiencies or emotional abnormalities are unmasked in a novel aversive environment (Burke et al., 2016). Furthermore, WKY rats demonstrate increased social avoidance (Paré, 2000; Pardon et al., 2002; Ferguson and Cada, 2004; Nam et al., 2014) and impaired recognition and spatial memory (Grauer and Kapon, 1993; Wyss et al., 2000; Langen and Dost, 2011; Gonzales et al., 2015; Shoval et al., 2016; Doharty, 2018; Willner et al., 2019).

Of importance, the WKY rats have a hyperalgesic phenotype. WKY rats show thermal hyperalgesia in the hot plate test (but not in the tail flick test) compared to SD rats (Burke et al., 2010). Greater nociceptive responding following intraplantar formalin injection, a noxious inflammatory stimulus, has also been reported in the WKY rats (Burke et al., 2010; Rea et al., 2014). Several studies have demonstrated visceral hypersensitivity in the WKY rats (Gunter et al., 2002; Greenwood-Van Meerveld et al., 2005; Gibney et al., 2010; O'Mahony et al., 2010; Johnson et al., 2012; O' Mahony et al., 2013). Moreover, WKY rats exhibit mechanical hypersensitivity under baseline (naïve) conditions (Taylor et al., 2001; Hestehave et al., 2019a) and following peripheral nerve injury (Zeng et al., 2008) and complete Freund's adjuvant injection into the temporomandibular joint (Wang

et al., 2012). Also, repeated forced swim stress can reduce formalin-evoked nociceptive behaviour in the WKY rats, compared to SD rats (Jennings et al., 2016). Furthermore, exposure to water avoidance stress can enhance urinary bladder hyperalgesia in the WKY rats (Robbins et al., 2007; Lee et al., 2015), perhaps due to reduced glutamate clearance in the spinal cord (Ackerman et al., 2016). A recent study has shown that baseline anxiety-like behaviour in the WKY rats is predictive of augmented nociceptive response (mechanical allodynia) in the monosodium iodoacetate model of knee osteoarthritis pain (Burston et al., 2019). Taken together, these studies strongly indicate that the WKY rats represent a useful preclinical model for studying the influence of genetic background on the interrelationship between negative affect and pain.

The genetics underlying the characteristic phenotype of the WKY rat model is, as yet, unclear. Microarray analysis has revealed differences in the expression of several genes in the LC and dorsal raphe (the main noradrenergic and serotonergic nuclei, respectively, in the brain) between WKY and SD rats. WKY rats have higher expression of the genes encoding for the enzymes involved in noradrenaline turnover in the LC (Pearson et al., 2006). In the dorsal raphe nucleus, genes encoding for several potassium channels and cytoskeletal proteins for structural plasticity are lower in the WKY rats, compared with SD counterparts (Pearson et al., 2006). Furthermore, the expression of mRNA encoding catechol-O-methyltransferase (COMT, an enzyme involved in catecholamine degradation) is 4-7 fold higher in the cerebral cortex of WKY rats compared to SD rats (Walker et al., 2004). Thus, it is possible that a dysfunction in the monoaminergic system in the WKY rat strain may underlie its genetic predisposition to anxiodepressive-like behaviours.

Although the WKY is classed as an inbred rat strain and, as such, genetic homogeneity is assumed, several studies have revealed that this may not be the case. There is evidence of genetic heterogeneity in the WKY rat strain supplied commercially by different vendors, thus confounding results that may account for behavioural and neurochemical differences seen across similar studies that use different vendors. Zhang-James and colleagues have reported that WKY rats from Charles River (WKY/NCrl) show an overall 2.64% genetic difference in the form of single nucleotide polymorphisms (SNPs) from the WKY rats obtained from Harlan (now Envigo, WKY/NHsd) (Zhang-James et al., 2013). The WKY rats, which were bred to F10 generation in Kyoto School of Medicine, were sent to the National Institute of Health (NIH) in 1971 (Kurtz et al., 1989). NIH then distributed the

WKY breeding stock first to Charles River Laboratories in 1971 and much later to Harlan/Envigo in 1982 (Kurtz et al., 1989). Hence, genetic drift as a result of maintenance policies of the WKY lines by individual vendors (Browne et al., 2015) may account for such genetic variability between the WKY rats from these two suppliers.

WKY rats from Charles River, Envigo, and Taconic also exhibit differences at the behavioural level. For instance, WKY/NHsd rats display three times greater behavioural variability compared to other in- and outbred rat strains (Will et al., 2003). WKY/NCrl rats though not hyperactive or impulsive, show deficits in sustained attention whereas WKY/NHsd rats are not hyperactive, impulsive, or inattentive (Sagvolden et al., 2008). In addition, the authors found substantial genomic differences between the WKY/NCrl and WKY/NHsd with large stretches of divergence in the form of SNPs on chromosomes 1, 2, and 3 (Sagvolden et al., 2008). Moreover, WKY/NCrl rats develop greater stress-induced ulcer than WKY/NHsd rats (Paré and Kluczynski, 1997). In addition, WKY/NCrl and WKY/NHsd rats show high baseline immobility in the forced swim test compared to WKY rats from Taconic (Browne et al., 2015). Furthermore, WKY/NCrl rats display greater hypolocomotor activity than WKY/NHsd rats when exposed to a novel aversive environment in the open field test (van Zyl et al., 2014). In the literature, the WKY rats from all suppliers continue to be used equally and often interchangeably which may affect interpretation of results from the studies. Hence, it is extremely important to choose a supplier and indeed continue with the same supplier when comparing across studies (Zhang-James et al., 2013).

1.3.4. Neural substrates implicated in hyperalgesia associated with negative affect

There is considerable overlap in the regions within the CNS that are important in processing stress, affect, and pain. In turn, nociceptive signalling can be influenced by stress and negative affect (Jennings et al., 2014). Persistent stress and/or negative affective states may result in maladaptive neuroplastic changes in many of these key regions, resulting in altered neuronal activity and hence exacerbated pain perception. The following sections will review the role of these key supraspinal and spinal components in mediating hyperalgesia associated with stress or negative affect.

1.3.4.1. Prefrontal cortex

The PFC is not only important in executive functions, but also in the processing of both sensory and affective dimensions of pain experience (Ong et al., 2019). The medial PFC

(mPFC) is composed of multiple nuclei, including the infra- and prelimbic and anterior cingulate cortices. Human neuroimaging studies have consistently shown reduced volume of the mPFC in different types of chronic pain (Apkarian et al., 2004; Schmidt-Wilcke et al., 2005; Blankstein et al., 2010; Niddam et al., 2017). In addition to morphological changes, hyperactivity of PFC, particularly increased activity in the ACC, has been reported in patients with chronic pain (Apkarian et al., 2001). Also, a positive correlation has been reported between enhanced pain sensitivity in patients with chronic pain and increased mPFC activity (Baliki et al., 2006). Nevertheless, how stress and negative affect mediates alterations in prefrontal cortical activity in patients with chronic pain remains unclear. Some studies have shown that healthy volunteers experience increased pain sensitivity to electrical stimulation in the presence of a negative context, which has been associated with increased pain-related activation of the ACC (Yoshino et al., 2010, 2012). An imaging study in patients with irritable bowel syndrome exposed to a psychological stressor has reported that enhanced visceral pain sensitivity is associated with increased activation of several brain regions including the mid-cingulate and ventrolateral PFC, compared to healthy subjects (Elsenbruch et al., 2010). Hence, both morphological and functional changes in the PFC may contribute to the interaction between stress and pain.

Similar to those observed in clinical populations, studies in rat models of chronic pain suggest morphological and functional reorganisation of the mPFC, particularly the ACC (Metz et al., 2009; Seminowicz et al., 2009; Kiritoshi and Neugebauer, 2015). Electrical stimulation and optogenetic activation of the ACC facilitates spinal nociception (Zhang et al., 2005a; Chiou et al., 2016) whereas lesioning this region prevents nociception-related aversion (Johansen et al., 2001; Qu et al., 2011), indicating that the ACC is important in mediating enhanced nociception as well as the negative affective component of pain. SIH was associated with increased expression of c-Fos, a marker of neuronal activity, in the PFC in rats (Felice et al., 2014; Jain et al., 2015). In a rat model of chronic inflammatory pain, persistent stress further exaggerated allodynia and concurrently increased expression of phosphorylated extracellular signal-regulated kinase (ERK) in the mPFC (Qi et al., 2014). Moreover, levels of the excitatory neurotransmitter glutamate were enhanced in the PFC in a mouse model of neuropathic pain exhibiting comorbid anxiety- and despair-like behaviours (Gonzalez-Sepulveda et al., 2016). These studies indicate that abnormal activity of the PFC may play a role in hyperalgesia induced by stress and negative affect. Furthermore, lesioning of the prelimbic cortex attenuated

complete Freund's adjuvant-induced hyperalgesia and anxiety-like behaviours in rats (Wang et al., 2015b). The PFC is also extensively interconnected with the limbic brain structures (Millan, 2002). Hence, there is a possibility that alteration in this cortical structure may affect neuronal activity in downstream regions and contribute to SIH (Liu et al., 2019a).

1.3.4.2. Amygdala

The amygdala is an important neural substrate of the limbic brain system and plays a prominent role in the interaction between pain and emotion/affect (Neugebauer et al., 2004). Neuroimaging studies have demonstrated reduced volume and aberrant functioning of the amygdala in patients with chronic pain (Labus et al., 2014; Simons et al., 2014b; Mao and Yang, 2015; Vachon-Presseau et al., 2016). Increased functional connectivity in amygdala-PFC circuitry was also identified as a risk factor for the persistence of back pain (Hashmi et al., 2013; Vachon-Presseau et al., 2016). In addition, chronic stress was associated with increased activity of the amygdala in a cohort of healthy subjects (Rosenberger et al., 2009).

In agreement with clinical findings, stress-induced visceral hypersensitivity has been shown to correlate positively with functional activity of the amygdala in rats (Wang et al., 2013b; Holschneider et al., 2016). Additionally, a few studies have reported that stress-induced exacerbation of neuropathic pain is associated with increased neuronal activity of the amygdala in rats (Ait-Belgnaoui et al., 2009; Nishinaka et al., 2016). Direct injection of corticotropin-releasing factor (CRF) into the central nucleus (CeA, a subnucleus of amygdala) results in visceral hypersensitivity in rats (Su et al., 2015). The CRF in the brain has been implicated in stress-related affective disorders (Reul and Holsboer, 2002). Pharmacological blockade of the CRF-1 receptor in the CeA has been shown to prevent development of allodynia and anxiety-like behaviour in a rat model of arthritis pain (Ji et al., 2007). Moreover, a recent study has shown that chronic forced swim stress-induced augmentation of allodynia in a rat model of neuropathic pain is associated with neuroplastic changes and sensitisation of CeA neurons (Li et al., 2017), further indicating a key role of this amygdalar nucleus in SIH.

1.3.4.3. Hippocampus

Another key limbic brain region is the hippocampus, which has a well-established role in cognition (Fanselow and Dong, 2010). The hippocampus has also emerged as a critical

region in emotionality, particularly anxiety and depression, and comorbid pain (Barkus et al., 2010; Mutso et al., 2012; Liu et al., 2017). Abnormal hippocampal activity has been reported in patients with chronic pain (Niddam et al., 2008; Maleki et al., 2013; Vachon-Presseau et al., 2013). Hippocampal volume is decreased in patients with depression (Chan et al., 2016) and chronic low back pain (Mutso et al., 2012; Vachon-Presseau et al., 2013). In line with this, dendritic atrophy within the hippocampus has been shown in a rat model of chronic stress with associated hyperalgesia (Kim et al., 2013). Reduced expression and phosphorylation of ERK and synaptic plasticity was reported in rodent models of neuropathic pain and comorbid depression, further suggesting involvement of hippocampus in learning and emotional deficits observed in chronic pain (Mutso et al., 2012; Wang et al., 2015d). Furthermore, some studies have reported alterations in glial cell activity (astrocytes and microglia) and increased levels of inflammatory mediators (particularly tumour necrosis factor alpha) in the hippocampus in rat models of SIH with comorbid anxiety and/or depression (Burke et al., 2013b; Sun et al., 2016a; Zhao et al., 2019). Taken together, aberrant changes in the structure, function, and connectivity of the hippocampus with other brain regions could contribute stress/affect-pain interaction.

1.3.4.4. Periaqueductal grey

The midbrain PAG is a key component of the descending pain pathway. It can be anatomically and functionally divided into 4 subregions along the rostro-caudal axis: dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG), and ventrolateral (vlPAG) columns. Each PAG column receives a distinctive set of ascending and descending afferents, thus mediating distinct emotional coping strategies (active and passive) to different types of stress and allowing the organism to respond appropriately to potential threats and noxious stimuli (Keay and Bandler, 2001). Despite its key role in modulating nociceptive and aversive behaviours, neuroimaging study of SIH with regard to the PAG in humans is scarce. Only one study to date has shown that chronic stress (rated on the perceived stress questionnaire) is correlated with activation of the PAG during visceral stimulation in healthy female volunteers (Rosenberger et al., 2009). The authors have suggested that chronic stress may alter pain control from the PAG, resulting in increased enhanced pain.

Animal studies have demonstrated that lesioning the vlPAG subcolumn attenuates formalin-evoked nociceptive behaviour but does not affect anxiety-like behaviour in the elevated plus maze test (Mendes-Gomes et al., 2011). Furthermore, early life stress can

affect functional activity of a number of brain regions including the PAG in adult rats (Holschneider et al., 2016). In this regard, Wouter and colleagues have studied brain responses to noxious visceral stimulation in a rat model of early life stress (maternal separation, MS) before and after an acute stress (water avoidance, WA) challenge (Wouters et al., 2012). The authors found that MS rats displayed increased PAG activation during noxious visceral stimulation both pre- and post-WA stress. However, MS rats exposed to WA stress also showed increased activation of the somatosensory cortex, compared with pre-stress activity. Based on these findings, the authors suggested that enhanced visceral hypersensitivity in the MS rats post-WA stress may be due to increased pain perception rather than an alteration in descending pain inhibition by the PAG, as the acute stress challenge did not further affect PAG activity to noxious stimulus. Of note, this study did not include a non-MS cohort which makes it difficult to fully interpret the data in the context of MS-induced changes. In a series of studies, Devall and coworkers reported that exposure to anxiogenic vibration stress induced hyperalgesia in only those female Wistar rats that were at late dioestrous stage (Devall and Lovick, 2010; Devall et al., 2011). This was accompanied by a robust increase in c-Fos expression in the PAG (which was not seen in other cycle stages), indicating an enhanced PAG activity in response to changes in gonadal hormone levels. Thus, the PAG may be involved in mediating the oestrous cycle-linked hyperalgesia in the setting of mild anxiety.

Chronic restraint stress induces allodynia that was associated with a decrease in the expression of glial fibrillary acidic protein (GFAP, a marker for astrocytes) and excitatory amino acid transporter 2 (a glutamate transporter protein) in the PAG (Imbe et al., 2012). However, expression of GFAP was upregulated in the rat spared nerve injury (SNI) model of neuropathic pain (Norman et al., 2010). Moreover, exposure to chronic restraint stress before nerve injury exacerbated mechanical allodynia and depression-like behaviours and additionally increased expression of brain derived neurotrophic factor (BDNF) in the PAG, compared to non-stress counterparts (Norman et al., 2010). These findings suggest that stress-induced behavioural responses are possibly due to alterations in the PAG level of various neuromodulators that are involved in neural plasticity, but the directionality of changes may depend on the type of model used. A recent RNA sequencing study has reported that SNI and chronic unpredictable stress induce changes in gene expression patterns in the PAG that belong to common biological signal transduction pathways and show robust overlap with the genes previously implicated in anxiodepressive states and

pain in the clinical population (Descalzi et al., 2017). Taken together, stress-induced alterations in the neurobiology of the PAG may contribute to changes in the activity of the descending inhibitory pain pathway to facilitate hyperalgesia.

1.3.4.5. Rostral ventromedial medulla

The RVM is the final supraspinal relay station and a major component of the descending inhibitory pain pathway. It contains ON and OFF cells (Fields et al., 1983a; Heinricher et al., 1992a, 1994) that are involved in descending facilitation and inhibition of nociceptive signal, respectively, and it projects to the DHSC and trigeminal nucleus to exert bidirectional control over nociception (Heinricher et al., 2009). The RVM Neutral cells represent a third subset, but their exact physiological function is not yet known (Heinricher et al., 2009). Evidence from preclinical studies indicates a key role of the RVM in SIH. Repeated restraint stress-induced hyperalgesia is associated with a decrease in GFAP (Imbe et al., 2013) and an increase in phosphorylated ERK (Imbe et al., 2004) in the RVM. In addition, Imbe and colleagues has reported an increase in acetylation of histone H3 in GABAergic, but not serotonergic, neurons in the RVM using the same model of SIH (Imbe and Kimura, 2018). Also, MS-induced hyperalgesia is associated with an increase in the expression of tyrosine kinase receptor B but not its ligand BDNF in the RVM in rats (Chung et al., 2009).

Chemical lesion or inactivation of the RVM using ibotenic acid or lidocaine, respectively, attenuates SIH in rats (Imbe et al., 2010). Furthermore, air puff-induced hyperalgesia is prevented by selectively blocking the RVM ON cells with excitatory amino acid antagonist kynurenate (Martenson et al., 2009). In addition, direct administration of the cholecystokinin (CCK)-2 receptor antagonist, CI-988, into the RVM reverses chronic social defeat stress-induced mechanical hypersensitivity (Rivat et al., 2010). The CCK system has been implicated in descending pain facilitation and anxiety (Hebb et al., 2005). In summary, these studies suggest stress-induced neural plasticity in the RVM may result in top-down facilitation of pain.

1.3.4.6. Spinal cord

The effect of stress and mechanisms underlying SIH at the level of spinal cord is poorly understood. Preclinical animal studies suggest stress-induced alterations in spinal circuitry can potentially perturb the balance between inhibitory and excitatory neurotransmission in the spinal cord. Some studies have demonstrated that SIH is

associated with increased expression of c-Fos in the lumbar spinal cord and DRG in rats (Quintero et al., 2003, 2011; Tsang et al., 2012; Qi et al., 2016), indicating enhanced neuronal activity in spinal circuitry. Quintero et al have also reported that repeated forced swim stress enhances formalin-evoked nociceptive response in rats, which is associated with decreased GABA but increased glutamate levels in the lumbar spinal cord (Quintero et al., 2011). Moreover, blocking the *N*-methyl-d-aspartic acid (NMDA) glutamate receptor prior to stress exposure prevented SIH and changes in spinal glutamate levels (Suarez-Roca et al., 2006; Quintero et al., 2011), suggesting a possible role for spinal glutamate transmission in mediating SIH. In addition, upregulation in glial cell activation (particularly microglia) in the spinal cord has been reported in several rodent models of SIH (Akagi et al., 2014; Suarez-Roca et al., 2014; Qi et al., 2016; Sun et al., 2017; Mizoguchi et al., 2019), indicating a possible crosstalk between neurons and activated glial cells in the spinal cord that may contribute to a stress-induced hyperactivated state, leading to increased pain sensitivity. Others have shown alterations in BDNF levels in the spinal dorsal horn, although the direction of change may be species-dependent (Winston et al., 2014; Huang et al., 2019). Together, these findings suggest that stress can affect neurobiology of spinal cord circuitry. Thus, alterations in spinal signalling, mediated either via direct effects of stress or via top-down (indirect) stress-induced modulation from supraspinal sites, play a key role in mediating SIH.

1.4. Endogenous opioid system

Opium, an extract of the opium poppy plant (*Papaver somniferum*), has been used for medicinal and recreational purposes for centuries. Its use to relieve pain dates to as early as 3400 BC (Breasted, 1930). In the early 19th century, the first naturally occurring alkaloid in opium was isolated by the German pharmacist Serturner and was named morphine (Schmitz, 1985). Subsequent pharmacological studies with morphine demonstrated that its analgesic effect was similar to that produced by electrically stimulating the PAG (Akil et al., 1972), giving rise to the concept of particular receptive structures for opioids in cells and tissues. Both morphine-induced and stimulation-produced antinociception could be blocked by naloxone (Akil et al., 1976), further implicating that both these phenomena were mediated by the same endogenous system. The existence of an endogenous opioid system was further substantiated by the discovery of specific opioid-binding sites in the brain in 1973 (Pert and Snyder, 1973). Shortly after the identification of the receptors, enkephalin was isolated in mammalian brain and

characterised as the first endogenous ligand for these sites (Hughes, 1975; Simantov and Snyder, 1976).

1.4.1. Opioid receptors

The current understanding is that the endogenous opioid system is comprised of three opioid receptors, μ (mu or MOP), κ (kappa or KOP), and δ (delta or DOP), which are also sometimes referred to as the classical opioid receptors. Characterisation of orphan receptors using molecular cloning methodology led to the identification of a fourth type of opioid receptor, nociceptin/orphanin FQ receptor [NOP, also referred to as opioid receptor-like receptor-1 (ORL-1) initially]. NOP shares a high degree of structural homology (~60%) with the classical opioid receptors (Mollereau et al., 1994). All four opioid receptors have been cloned. The genes encoding the opioid receptors – *OPRM1* (Min et al., 1994; Wang et al., 1994b; Zastawny et al., 1994), *OPRK1* (Meng et al., 1993; Liu et al., 1995; Simonin et al., 1995), *OPRD1* (Kieffer et al., 1992; Abood et al., 1994; Knapp et al., 1994), and *OPRL1* (Bunzow et al., 1994; Wang et al., 1994a) – have been identified and their chromosomal locations pinpointed (Befort et al., 1994; Mollereau et al., 1994; Wang et al., 1994b; Yasuda et al., 1994). In addition, high-resolution crystal structures of the opioid receptors have been reported, further facilitating understanding of receptor-ligand interaction (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012).

1.4.2. Opioid peptides

The endogenous ligands for the opioid receptors are derived from large precursor proteins, namely proopiomelanocortin (POMC), prodynorphin (PDYN), proenkephalin (PENK), and pronociceptin (PNOC). Following complex posttranslational modifications, POMC yields several active peptides, of which the major opioid peptide is β-endorphin that shows affinity for MOP and to a lesser degree for DOP (Li and Chung, 1976; Raynor et al., 1994). PDYN is cleaved to dynorphins (including dynorphins A and B, neodynorphins, and big dynorphin), which have preferential affinity for the KOP (Chavkin et al., 1982). Met- and Leu-enkephalins are derived from PENK, both of which bind preferentially to DOP but also display high affinity for MOP (Lord et al., 1977; Kosterlitz, 1985). PNOC is the precursor of the peptide, nociception/orphanin FQ (N/OFQ), which acts via the NOP (Meunier et al., 1995; Reinscheid et al., 1995). All opioid peptides contain a common N-terminal tetrapeptide sequence [Tyr-Gly-Gly-Phe-

(Met or Leu)] referred to as an "opioid motif" that is slightly modified in N/OFQ. As a result, the endogenous ligand N/OFQ shows a high degree of selectivity for NOP over the three classical opioid receptors (Meunier, 1997). Another group of endogenous peptides called the endomorphins (endomorphin-1 and endomorphin-2) have also been isolated. Endomorphins demonstrate high affinity and remarkable selectivity for MOP (Zadina et al., 1997) but their synthetic pathway *in vivo* is yet to be elucidated. The DNA sequence of a novel protein, named proMexneurin, has been identified recently from the whole mouse brain cDNA library using immunoscreening technique (Matus-Ortega et al., 2017). ProMexneurin contains a peptide sequence similar to an endomorphin-like peptide and, hence, has been proposed as a candidate for precursor of endomorphins. The opioid peptides are rapidly degraded by hydrolysis, predominantly by two enzymes: aminopeptidase N and neutral endopeptidase (also known as enkephalinase) (Schwartz et al., 1981; Hersh, 1985; Waksman et al., 1986; Noble et al., 2001).

1.4.3. Anatomical distribution

The opioid receptors are present abundantly on pre- and postsynaptic neurons throughout the CNS. Each of the opioid receptors show overlapping but distinct anatomical distribution in the CNS (Figure 1.3) as demonstrated by autoradiography, *in situ* hybridisation, immunohistochemistry, and neuroimaging studies in rodents and humans (Mansour et al., 1995; Melichar et al., 1997; Le Merrer et al., 2009; Lutz and Kieffer, 2013). All four opioid receptors are expressed in the cortex, limbic regions, brainstem, DRG, and spinal dorsal horn (Le Merrer et al., 2009). The MOP are densely expressed in the neocortex, throughout the midbrain and subcortical regions, including the insula, thalamus, and PAG (Mansour et al., 1995; Zubieta et al., 2001; Le Merrer et al., 2009). The MOP are also moderately expressed in the amygdala, hippocampus, and striatum. High expression of KOP is found in the PFC and limbic brain areas such as the amygdala, hippocampus, and hypothalamus (Simonin et al., 1995; Le Merrer et al., 2009; Lutz and Kieffer, 2013). The KOP are found at moderate level in the PAG and RVM (Gutstein et al., 1998). The DOP are expressed abundantly in the frontal cortex, amygdala, and striatum and, to a moderate extent, in the hippocampus (Scherrer et al., 2006; Pradhan et al., 2011). Compared to the expression of MOP and KOP, the distribution of DOP seems more restricted since their expression is low in the hypothalamus, midbrain, and brainstem (Le Merrer et al., 2009). High level of NOP expression is found in the cortex and limbic structures including amygdala (Neal et al., 1999; Berthele et al., 2003; Lohith

et al., 2012; Ozawa et al., 2015). The opioid receptors are also expressed in the main monoaminergic nuclei of the brain: dorsal raphe nucleus, LC, and ventral tegmentum area, from where the neurons for the serotonergic, noradrenergic, and dopaminergic systems, respectively, originate and project to several brain regions, including the PFC, hippocampus, and amygdala (Le Merrer et al., 2009; Lutz and Kieffer, 2013). Additionally, opioid receptors are located on glial cells (astrocytes and microglia) (Ruzicka et al., 1995; Chao et al., 1996), as well as neurons in peripheral tissues (Stander et al., 2002; Salemi et al., 2005), immune cells (Kapitzke et al., 2005), and other organs (Bagnol et al., 1997).

Distribution of opioid peptides in the CNS largely overlaps with the localisation of opioid receptors. PDYN, PENK, PNOC are expressed in local neurons at multiple levels distributed throughout the CNS, including the cortex, hippocampus, hypothalamus, PAG, RVM, and DHSC (Le Merrer et al., 2009; Benarroch, 2012a). In contrast, the POMC-expressing neurons are more restricted to two locations, arcuate nucleus of the hypothalamus and brainstem nucleus tractus solitarius, which have projections to the limbic regions, midbrain, brainstem, and spinal cord (Le Merrer et al., 2009; Benarroch, 2012a). In some regions, opioid peptides are coexpressed with other neurotransmitters, such as glutamate and GABA (Renno et al., 1999; Huang et al., 2008; Marvizon et al., 2009). Similar to opioid receptors, non-neuronal tissues particularly immune cells also contain opioid peptides (Kapitzke et al., 2005). In summary, the widespread distribution of opioid receptors and peptides in supraspinal, spinal, and peripheral sites positions the endogenous opioid system for a key role in the regulation of a range of physiological processes, including (but not limited to) nociceptive signalling, stress response, mood/emotion, aversion, and cognition.

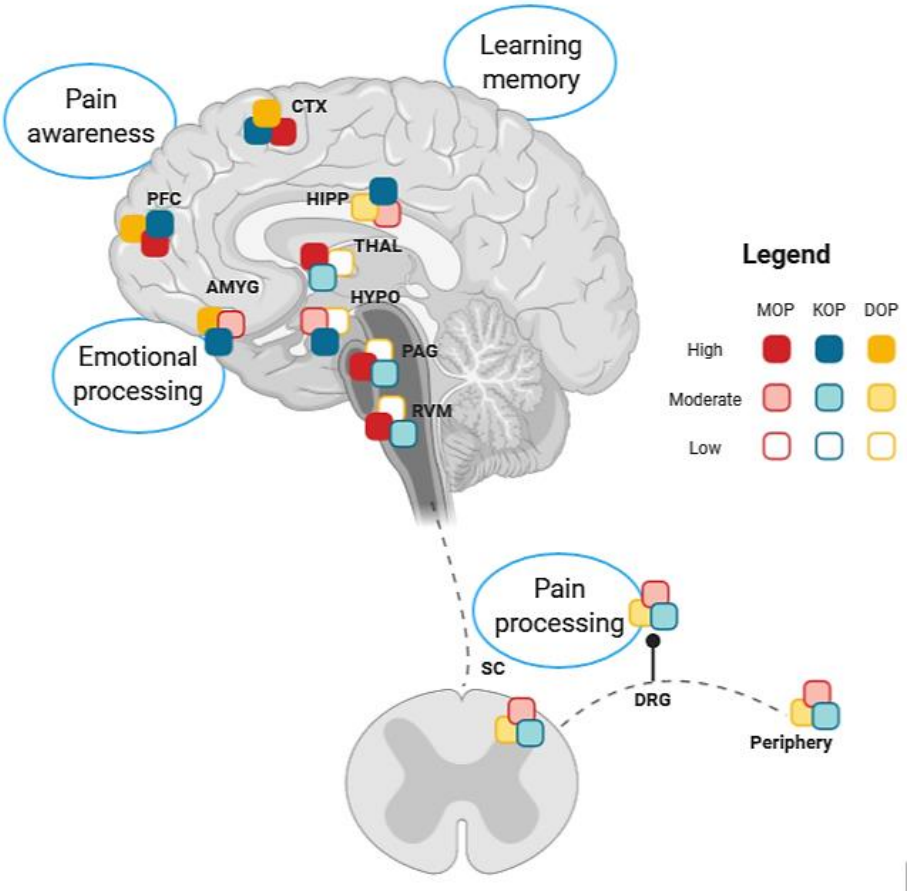


Figure 1.3: Anatomical distribution of the three classical opioid receptors – MOP, KOP, and DOP (in red, blue, and yellow, respectively) – in neural substrates implicated in pain, affect, and cognition. The filled, shaded, and open squares indicate high, moderate, and low receptor expression, respectively. AMYG: amygdala, CTX: cortex, DOP: delta-opioid receptor, DRG: dorsal root ganglia, HIPP: hippocampus, HYPO: hypothalamus, KOP: kappa-opioid receptor, MOP: mu-opioid receptor, PAG: periaqueductal grey, PBN: parabrachial nucleus, SC: spinal cord, THAL: thalamus, RVM: rostral ventromedial medulla. Adapted and modified from (Le Merrer et al., 2009; Pradhan et al., 2011; Lutz and Kieffer, 2013; Browne and Lucki, 2019).

1.4.4. Opioid signalling mechanism

All four opioid receptors belong to the superfamily of seven-transmembrane spanning G-protein-coupled receptors (GPCR), coupled to pertussis toxin sensitive heterotrimeric inhibitory G-protein (G_i/G_o) (Al-Hasani and Bruchas, 2011) (Figure 1.4). Activation of opioid receptors by an endogenous (e.g. opioid peptides) or exogenous (e.g. morphine) agonist results in dissociation of the Gα and Gβγ subunits from each other, subsequently leading to a cascade of intracellular events. The $G\alpha_i$ subunit mediates inhibition of adenylyl cyclase (reducing cyclic adenosine monophosphate (cAMP) levels in the cell) (Ho et al., 1973; Attali and Vogel, 1986; Taussig et al., 1993) and rapid activation of inwardly rectifying potassium (K^+) channels (Williams et al., 1982; North and Williams, 1985; Meunier, 1997). The Gβγ subunit mediates inhibition of presynaptic voltage-gated calcium (Ca^{2+}) channels, thus reducing Ca^{2+} conductance (Mudge et al., 1979; Seward et al., 1991; Meunier, 1997; Rusin et al., 1997). Moreover, because activation of opioid receptors blocks adenylyl cyclase activity, the cAMP-dependent Ca^{2+} influx is also reduced. At the cellular level, the net effects are inhibition of presynaptic calcium-dependent neurotransmitter release and hyperpolarisation of postsynaptic neuronal membranes, consequently reducing overall neuronal excitability (Al-Hasani and Bruchas, 2011).

Opioid receptor activation may also recruit other intracellular second messenger systems including phospholipase C/protein kinase C (Murthy and Makhlouf, 1996) and phosphoinositide-3-kinase/protein kinase B (Olianas et al., 2011) pathways. Following agonist-induced activation, signalling through opioid receptors is regulated by desensitization of the receptors, which is a complex feedback regulatory process involving phosphorylation of the receptors by G-protein-coupled receptor kinases and binding of β-arrestin to the receptors. This interferes with further G-protein coupling and facilitates receptor internalization, which is then followed by recycling or degradation via endocytosis (Law et al., 2000). Phosphorylated-arrestin bound opioid receptor complex can also recruit alternate signal transduction cascades such as the mitogen activated protein kinases (MAPK) (Fukuda et al., 1996; Zhang et al., 1999; Bruchas et al., 2006; Melief et al., 2010). Thus, there are various functional consequences of opioid receptor activation that are dependent on the differential expression and strategic localisation of the different opioid receptors and peptides in discrete neural substrates. Moreover, the fact that these opioid receptors not only share some common signalling pathways but can

also recruit multiple second messenger systems enables them to regulate and modulate many aspects of cellular events, physiology, and behaviour.

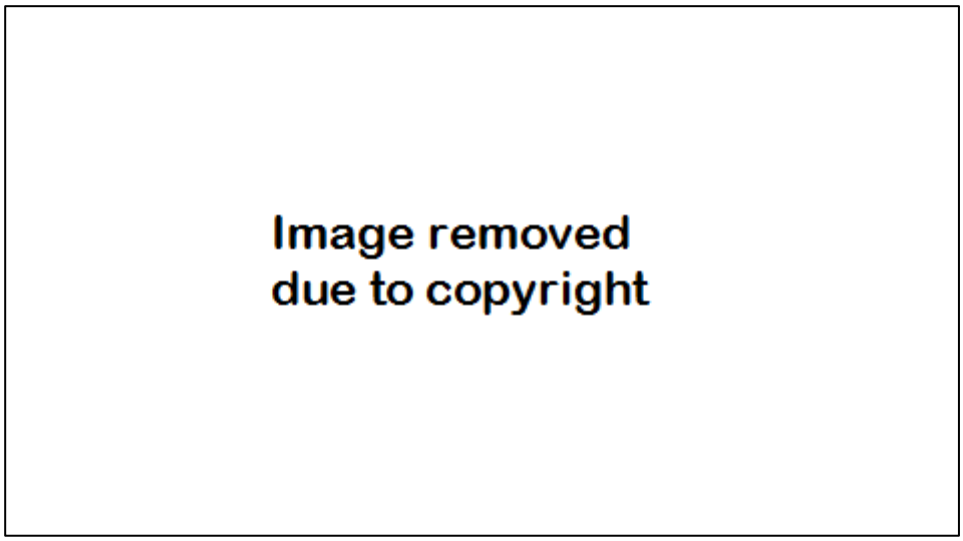


Figure 1.4: Opioid receptor signalling. In general, all opioid receptors share these common signalling pathways. Agonist-induced activation of GPCR leads to a cascade of intracellular events, which result in an increase in K^+ and a decrease in Ca^{2+} conductance, consequently reducing overall neuronal activity of both pre- and postsynaptic neurons. Desensitisation of the receptor involves phosphorylation and binding of β-arrestin, resulting in internalisation, which is followed by degradation or recycling of the receptor complex or further recruitment of MAPK signalling pathways. *Arrows* refer to activation steps; *T lines* refer to blockade or inhibition of function. βγ: G protein βγ subunit, cAMP: cyclic adenosine monophosphate, Ca^{2+}: calcium, G_i: inhibitory G-protein, ERK: extracellular signal-regulated kinase, JNK: c-jun N-terminal kinase, K^+: potassium, MAPK: mitogen-activated protein kinases, P: phosphorylation. Adapted from (Al-Hasani and Bruchas, 2011).

1.4.5. Endogenous opioid system and pain

As described previously, the opioid receptors and peptides are located extensively at all levels of the descending pain pathway and are key components in modulating the experience of pain (Ossipov et al., 2010; Benarroch, 2012a). Pharmacological manipulation of the opioid system has confirmed its involvement in the inhibition of nociceptive signalling. The activation of all three classical opioid receptors (MOP, KOP,

and DOP) produces antinociceptive effect (Kivell and Prisinzano, 2010; Pradhan et al., 2011; Corder et al., 2018). Direct microinjection of opioids (e.g. morphine) into discrete brain regional components of the descending pain pathway produces antinociception (Tsou and Jang, 1964; Basbaum et al., 1976; Yaksh et al., 1976b; Lewis and Gebhart, 1977; Yeung et al., 1977; Porreca et al., 1987; Manning et al., 1994; Helmstetter et al., 1998), effects that can be blocked by naloxone (Akil et al., 1976), a nonselective opioid receptor antagonist (Blumberg et al., 1961). Moreover, lesions of the RVM or amygdala disrupt the antinociceptive effects of morphine (Fields et al., 1976; Matthies and Franklin, 1992; Manning and Mayer, 1995b, 1995a). Opioid-induced antinociception within descending pain modulatory circuits is mediated, at least in part, via supressing the tonic inhibitory influence of GABAergic interneurons within PAG and RVM, thereby disinhibiting the antinociceptive drive of the neuronal output to the spinal dorsal horn (Vaughan et al., 1997; Lau and Vaughan, 2014). In addition, opioid receptors present in the primary afferent neurons in peripheral tissues can mediate antinociceptive effects, particularly in the setting of inflammation, by inhibiting the release of excitatory neurotransmitters (e.g. substance P) from these terminals (Zachariou and Goldstein, 1996b, 1996a; Kondo et al., 2005). Like the classical opioid receptors, the NOP modulate nociception. However, the effects of activating NOP in spinal and supraspinal sites for modulating nociception in rodents are bidirectional (exhibiting both pronociception and antinociception) depending on the doses, assays, and pain modalities (Kiguchi et al., 2016).

Of note, many of the brain regions (such as PFC, amygdala, and PAG) within the descending pain pathway are involved in the modulation of stress response, emotion, and affect (McEwen, 2007; Caputi et al., 2019). As such, the opioid receptors have been shown to modulate these behavioural responses along with the regulation of nociceptive signalling. In general, activation of MOP and DOP enhances mood whereas that of KOP produces aversion and prodepressive or dysphoric state in humans and rodents (Lutz and Kieffer, 2013), which will be discussed in more detail in respective chapters. Hence, the endogenous opioid system has been identified as a potential mediator of SIH and alteration in this system may play a key role in the reciprocal interactions between pain and stress/negative affect. The following section (section 1.4.6) will provide an overview of the clinical and preclinical evidence to date supporting a role of the opioid system in this interaction.

1.4.5.1. Opioid-induced hyperalgesia

Opioid-induced hyperalgesia (OIH) is a state of nociceptive sensitisation caused by exposure to opioids. It is a paradoxical response whereby sustained exposure to opioids leads to hyperalgesia to certain painful stimuli (Roeckel et al., 2016; Lueptow et al., 2018). OIH has been implicated in the development of tolerance to chronic opioid treatment. The precise neurobiological mechanisms of OIH are yet to be elucidated. Some studies suggest that increased activation (facilitation) of the descending pain pathway by chronic morphine exposure produces neuroadaptations, particularly in the RVM (e.g. increase in the number of ON cells), that may result in hyperalgesia (Vanderah et al., 2001; Meng and Harasawa, 2007). Other proposed mechanisms include long-term potentiation of the C-fibre synapses on the dorsal horn (Klein et al., 2007; Drdla et al., 2009), aberrant spinal glutamatergic signalling (Zhao et al., 2012), increased descending serotonergic facilitatory drive via 5-HT_3 receptors (Vera-Portocarrero et al., 2007; Liang et al., 2011), and activation of glial cells (astrocytes and microglia) and subsequent downregulation of the K^+-Cl^- co-transporter (KCC2) in the spinal dorsal horn (Ferrini et al., 2013, 2017; Grace et al., 2015). Further understanding of the mechanisms that underlie OIH is necessary and may help to find better solution for the adverse effects associated with long-term usage of opioids.

1.4.6. Endogenous opioid system in hyperalgesia associated with stress/negative affect

1.4.6.1. Evidence from clinical studies

A limited number of studies have investigated the role of the endogenous opioid system in SIH in humans and the findings are rather inconclusive at this stage (Table 1.3). In early studies, β-endorphin levels were measured in the CSF or plasma of patients with comorbid chronic pain and depression using radioimmunoassay. These studies found elevated (Almay et al., 1978; Atkinson et al., 1983), reduced (Nappi et al., 1985), or no change (Davis et al., 1982; Beutler et al., 1987; Daly et al., 1987; France et al., 1987; France and Urban, 1991) in β-endorphin level in the patients compared to healthy subjects. The heterogeneity of the patient population studied in these reports could result in this inconsistency. In addition, real-life stressful events such as sitting an exam (Michelotti et al., 2000) or dental surgery (Hargreaves et al., 1983) have been shown to

elevate anxiety levels and pain sensitivity in subjects with no change in plasma β-endorphin levels.

Some studies using functional neuroimaging have explored central opioidergic activity in pain-negative affect interaction in humans. Using [^{11}C]diprenorphine, a nonselective radiotracer that binds with equal affinity to MOP, KOP, and DOP, Jones and coworkers have assessed receptor-binding potential in trigeminal neuralgia patients before and after surgery for pain relief (Jones et al., 1999). Following surgery, patients showed increased [^{11}C]diprenorphine binding in brain regions related to pain modulation such as the prefrontal, insular, and cingulate cortices and thalamus, which also correlated with reduced pain and anxiety/depression scores in the patients. In contrast, higher opioid receptor availability in the contralateral temporal cortex, measured using [^{18}F]fluoroethyl-diprenorphine, was associated with high depression scores in patients with complex regional pain syndrome (Klega et al., 2010). Another study employing MOP selective radiotracer [^{11}C]carfentanil has shown that healthy subjects with higher MOP availability in the prefrontal, insular, and anterior cingulate cortices are prone to negative emotions during stress (Tuominen et al., 2012). Furthermore, increased baseline MOP availability in the thalamus of patients with chronic low back pain is inversely associated with positive affect ratings in the patients (Martikainen et al., 2013). However, reduced MOP availability in the amygdala is correlated with symptoms of depression in fibromyalgia patients (Harris et al., 2007). These studies suggest a region-dependent effect of MOP in chronic pain comorbid with negative affect.

To our knowledge, only one study to date has investigated the genetic association between chronic pain and negative affect in the context of the opioid system. Max and colleagues collected DNA samples from a group of 280 patients with sciatica to identify any genetic polymorphism in chronic pain-induced anxiety and depression (Max et al., 2006). Following surgery for pain relief, patients whose pain scores reduced by more than 25% showed improvement in symptoms of anxiety and depression. Three single-nucleotide polymorphisms (SNPs) in *OPRM1* were identified linking pain and postoperative mood. However, the sample size used in the study was small, limiting the power of statistical analysis, and future studies with larger sample sizes are warranted.

Table 1.3: Summary of clinical studies investigating the role of the endogenous opioid system in SIH in humans. ↑: increase, ↓: decrease, ↔: no change.

Clinical pain state/Painful stimulus	Stressor/source of anxiety or depression	Behavioural response	Methodology	Changes in opioid system	Reference
Migraine headache	Major depression		Radioimmunoassay	↓ CSF β-endorphin	(Nappi et al., 1985)
Perceived pain score	Surgical stress	↑ pain sensitivity and anxiety levels during surgery	Radioimmunoassay	↔ plasma β-endorphin	(Hargreaves et al., 1983)
Mechanical pressure	Undertaking academic exam	↑ pain sensitivity and state anxiety levels	Radioimmunoassay	↔ plasma β-endorphin	(Michelotti et al., 2000)
Mild electric shocks	Affective illness	↓ pain sensitivity	Radioreceptor assay	↔ CSF β-endorphin	(Davis et al., 1982)
Somatic pain complaints	Depression		Radioimmunoassay	↔ plasma β-endorphin	(Daly et al., 1987)
Rheumatoid arthritis	Depressive symptoms			↔ plasma β-endorphin	(Beutler et al., 1987)
Chronic low back pain/sciatica	Major depressive disorder		Radioimmunoassay	↔ CSF β-endorphin	(France et al., 1987; France and Urban, 1991)

Chronic pain	Depressive symptoms		Radioimmunoassay	↑ plasma β-endorphin	(Atkinson et al., 1983)
Chronic pain	Self-reported depressive symptoms		Radioreceptor assay	↑ CSF β-endorphin	(Almay et al., 1978)
Chronic low back pain/sciatica		↓ pain and anxiety/depression scores after surgery	SNP markers and PCR	3 SNPs in *OPRM1* gene linked to pain and postoperative mood	(Max et al., 2006)
Trigeminal neuralgia		↓ pain sensitivity and anxiety levels after surgery	PET, [^{11}C]diprenorphine	↑ binding in prefrontal, insular, and cingulate cortices and thalamus	(Jones et al., 1999)
Complex regional pain syndrome		↑ depression scores	PET, [^{18}F]fluoroethyl-diprenorphine	↑ binding in contralateral temporal cortex	(Klega et al., 2010)
Fibromyalgia		↑ depression scores	PET, [^{11}C]carfentanil	↓ MOP binding in amygdala	(Harris et al., 2007)
Chronic low back pain/sciatica		↓ positive affect ratings	PET, [^{11}C]carfentanil	↑ MOP binding in thalamus	(Martikainen et al., 2013)

1.4.6.2. Evidence from preclinical studies

1.4.6.2.1. Pharmacological studies

A growing body evidence from preclinical studies has implicated the endogenous opioid system in mediating SIH (Table 1.4). Both naloxone and naltrexone (nonselective opioid receptor antagonists) reverse hyperalgesia induced by novel environment stress (Le Roy et al., 2011) and prolonged exposure to restraint (Huang and Shyu, 1987) or forced swim (Suarez-Roca et al., 2006) stress in rats, indicating SIH is mediated by the opioid system. Repeated stress in the form of restraint or maternal separation induces an overall reduction in opioid receptor density in the CNS, particularly in the cortex, hippocampus, and spinal cord (Bernardi et al., 1986; Dantas et al., 2005). It is postulated that stress can induce long-lasting alterations in intracellular signalling pathways that facilitate a pronociceptive response (Khasar et al., 2008; Dina et al., 2009). Hence, such changes in neural substrates within descending pain pathway may underpin SIH (Green et al., 2011). Indeed, a recent study has demonstrated how stress may alter the directionality of opioidergic signalling of DRG neurons (Guerrero-Alba et al., 2017). DRG neurons from naïve mice, when incubated with supernatants from inflamed (chronic colitis) mouse colons, exhibited a naloxone-sensitive decrease in neuronal excitability. However, incubation in colonic supernatants from mice which were exposed to both chronic colitis and repeated WA stress resulted in a paradoxical enhancement of DRG neuronal excitability that was unmasked by naloxone. The authors have suggested that in the presence of chronic stress, endogenous opioid signalling switches from an antinociceptive to a pronociceptive pathway in DRG neurons that leads to visceral hyperalgesia. This stress-induced switch of the endogenous opioid signalling may involve the G_s-Protein Kinase A and/or $G_{\beta\gamma}$-Phospholipase C excitatory pathways (Guerrero-Alba et al., 2017).

In addition, alteration in the opioid system has been demonstrated in rodent models of chronic pain with associated anxiety/depression-like behaviour. Rats displaying anhedonia three months after inducing neuropathic pain showed reduction in opioid receptor availability (measured using *in vivo* brain imaging) in the insular cortex and striatum, regions involved in nociception and emotion (Thompson et al., 2018). In addition, Narita and coworkers have reported reduced MOP and DOP, but increased KOP, activity (measured using agonist-induced [^{35}S]GTPγS binding assay) in the amygdala (Narita et al., 2006a) and frontal cortex (Narita et al., 2006b) in mice models of chronic pain exhibiting comorbid anxiety-like behaviour. These studies indicate

receptor-dependent and region-specific alterations in the opioid system in pain-stress/negative affect interaction. The following sections will further review the roles of specific opioid receptor-peptide systems in SIH.

1.4.6.2.1.1. Mu-opioid system

The MOP system is the most studied in the context of SIH and several studies have reported alterations in the expression and functionality of the MOP in SIH. Repeated cold water swim stress-induced thermal hyperalgesia is associated with increased MOP expression in the midbrain, brainstem, and spinal cord in mice (Kim et al., 2003). In contrast, reduction in MOP expression in the spinal cord and DRG has been shown in rat models of SIH (Wang et al., 2015c; Hormozi et al., 2018, 2019), indicating possible species difference in chronic stress-induced alterations in MOP. Reduced expression of MOP has also been reported in brain regions such as the insular cortex, striatum, and hippocampus, as well as in the spinal cord, in rodent models of neuropathic pain with associated anxiety and depression-like behaviour (Ferreira-Chamorro et al., 2018; Thompson et al., 2018). Furthermore, selective ablation of MOP containing neurons in the RVM prevents development of mechanical hypersensitivity in rats following repeated whisker pad stimulation stress, suggesting that MOP in the RVM play a role in mediating SIH (Reynolds et al., 2011).

Blocking the MOP before stress sessions prevents SIH in rats, further implicating MOP in the development of SIH (Suarez-Roca et al., 2006). Antinociceptive efficacy of systemically administered morphine is reduced in rodents following various chronic stress paradigms such as exposure to cold (Schlen and Bentley, 1980; Nishiyori et al., 2010), immobilization/restraint (Puglisi-Allegra et al., 1986; da Silva Torres et al., 2003; Gameiro et al., 2005, 2006), intermittent cold-water swim (Girardot and Holloway, 1984b), forced swim (Suarez-Roca et al., 2006), maternal separation (Kalinichev et al., 2001), and unpredictable mild stress (Ide et al., 2015). These studies suggest a maladaptive change in MOP during repeated exposure to stress that possibly leads to the development of hyperalgesia. Moreover, morphine injected intracerebrally, but not intrathecally, failed to reduce thermal and mechanical hyperalgesia induced by chronic intermittent cold stress in mice (Nishiyori et al., 2010; Mukae et al., 2015), indicating maladaptive alterations of supraspinal MOP (perhaps not spinal MOP) contribute to SIH.

Monoamines play a key modulatory role in opioidergic signalling. Pretreatment with a noradrenaline reuptake inhibitor rescues the antinociceptive effect of morphine in a murine model of SIH (Ide et al., 2015), suggesting that the lower efficacy of morphine could be due to reduced noradrenergic tone in SIH. In comparison, a serotonin reuptake inhibitor did not affect morphine efficacy in the same model, which was attributed to the selection of drug dose by the authors (Ide et al., 2015). However, serotonin may have a role in modulating MOP signalling in SIH. Nishiyori and coworkers has demonstrated that intracerebral morphine failed to attenuate SIH in mice and concomitantly did not affect the turnover ratio of serotonin and its metabolite (5-HIAA) in the spinal cord (Nishiyori et al., 2010). Turnover of serotonin is crucial for opioid-mediated antinociception via the descending pathway (Tao and Auerbach, 1994; Taylor and Basbaum, 2003). Hence, a loss of spinal serotonergic activation due to stress-induced alteration of supraspinal MOP may also contribute to the reduced efficacy of morphine in SIH. These studies also suggest that, from a therapeutic standpoint, drugs that can modulate both MOP and monoaminergic signalling may have beneficial effects in patients with comorbid stress and pain. Indeed, unlike morphine, tramadol (a MOP agonist and reuptake inhibitor of both serotonin and noradrenaline) retains its antinociceptive efficacy in SIH (Ide et al., 2015). Moreover, repeated treatment with tramadol has been shown to reverse both mechanical hypersensitivity and associated anxiety- and depressive-like behaviours in a rat model of neuropathic pain, further illustrating therapeutic potential of tramadol in the context of pain-negative affect comorbidity (Caspani et al., 2014). Overall, these studies reveal chronic stress results in a dysfunction and/or desensitisation of the MOP signalling pathway that is key in mediating SIH.

1.4.6.2.1.2. Kappa-opioid system

Changes in the expression of KOP in SIH are mostly similar to those for MOP described above. Repeated cold water swim stress-induced hyperalgesia is associated with increased expression of KOP in the brainstem, midbrain, and spinal cord in mice (Kim et al., 2003). In contrast, KOP expression is lower in the spinal cord and dorsal root ganglia in rats displaying enhanced postsurgical pain following chronic sleep disturbance stress, compared to non-stress control rats (Wang et al., 2015c). In line with increased KOP functionality in SIH (Narita et al., 2006a), the KOP agonist U50488 produces greater antinociception in mice subjected to repeated cold stress compared to the non-stressed

control group (Omiya et al., 2000). In addition, both prophylactic and therapeutic treatment with the KOP agonist SA14867 attenuate hypersensitivity to pressure in rats exposed to specific alternation of rhythm in an environmental temperature (SART) stress (Tsukahara-Ohsumi et al., 2011). Indeed, blockade of KOP with MR1452 augments restraint stress-induced hyperalgesia in rats (Pilcher and Browne, 1983). However, the KOP antagonist nor-BNI has no effect on hyperalgesia induced by chronic forced swim stress in rats (Suarez-Roca et al., 2006). Methodological differences, such as nociceptive tests (pressure versus thermal) used to assess the effect of antagonists in SIH, may account for the discrepancy here since stress may not influence different pain modalities to the same extent. In summary, these studies show KOP play a crucial role SIH and, therefore, further investigation is warranted to fully understand the underlying mechanisms.

1.4.6.2.1.3. Delta-opioid system

Only a few studies have investigated the DOP in SIH. Unlike expression of MOP and KOP, levels of DOP are reduced in the brainstem, midbrain, and spinal cord in mice exposed to repeated cold water swim stress (Kim et al., 2003). Moreover, chronic intermittent foot shock-induced hyperalgesia markedly reduces levels of Met- and Leu-enkephalin in rat whole brains (McGivern et al., 1983). Pharmacological studies have shown that the antinociceptive potency of a DOP selective agonist DPDPE is not affected in a mice model of SIH (Omiya et al., 2000). Similarly, the DOP selective antagonist naltrindole has no effect on SIH in rats (Suarez-Roca et al., 2006), suggesting a limited contribution of the DOP in SIH. A recent study has shown that the DOP agonist UFP-512 is effective in alleviating nociceptive (mechanical, heat, and cold) hypersensitivity and depressive-like behaviour associated with chronic neuropathic pain in mice (Polo et al., 2019). This suggests that the DOP could be a useful therapeutic target in comorbid chronic pain and depression.

1.4.6.2.1.4. Nociceptin/orphanin FQ-opioid system

Visceral hypersensitivity induced by partial restraint stress in rats is attenuated by systemic, but not by intracerebroventricular, administration of the endogenous peptide N/OFQ, suggesting a modulatory role of the spinal and/or peripheral NOP system in SIH (Agostini et al., 2009). However, work from Zhang and coworkers provides evidence for a role of central NOP in the modulation of SIH. The authors have reported that single prolonged stress, which mimics clinical post-traumatic stress disorder (PTSD)-like stress,

induces mechanical allodynia, thermal hyperalgesia, and anxiety-like behaviour in rats (Zhang et al., 2012, 2015), which is reversed by systemic administration of the NOP antagonist JTC-801 (Zhang et al., 2015). Moreover, JTC-801 reduces N/OFQ peptide levels in the serum and cerebrospinal fluid (CSF) and in brain regions such as the PAG and hippocampus (Zhang et al., 2015), which are important in modulating nociception, aversion, and memory. In addition, chronic JTC-801 administration reverse stress-induced increases in NOP mRNA and protein levels in the amygdala and PAG (Zhang et al., 2015). Thus, upregulation of NOP and its endogenous opioid peptide N/OFQ in the brain may mediate SIH in rodents, positioning the NOP system as a potential therapeutic target in comorbid PTSD and pain.

1.4.6.2.2. Genetic studies

There is a paucity of studies employing transgenic mice to examine the involvement of the endogenous opioid system in exacerbated nociceptive response induced by stress or negative affect (Table 1.4). One study by Negrete and coworkers has demonstrated that KOP and PDYN null mice exhibit enhanced mechanical allodynia and anhedonia, but reduced anxiety-like behaviour, compared with their respective wildtype littermates. This finding suggests that the KOP and its endogenous ligand dynorphin may have a complex modulatory role in chronic pain state and associated anxiodepressive states (Negrete et al., 2017). Paretkar and Dimitrov have recently conducted a series of experiments where they used chemogenetics and transgenic mice to selectively modulate activity of the enkephalinergic neurons in the CeA. Targeted chemogenetic activation of enkephalinergic neurons in the CeA produced robust antinociception and also reduced anxiety-like behaviour in a model of chronic neuropathic pain (Paretkar and Dimitrov, 2019). Furthermore, sustained chemogenetic activation of the CeA enkephalinergic neurons prevented the development of anxiodepressive-like behavior in mice with neuropathic pain (Paretkar and Dimitrov, 2019). Hence, future studies using genetic models is warranted to further elucidate the complex roles played by the opioid receptors in the interaction between pain and negative affect.

Besides the use of transgenic mice, a small number of studies have used the inbred Wistar Kyoto (WKY) rat strain to investigate the role of the opioid system in altered nociceptive response associated with stress and/or negative affect. Gene microarray analysis has shown higher expression of the KOP-encoding gene in the LC in the WKY rats versus SD rats (Pearson et al., 2006). Other studies have revealed higher expression of KOP and

dynorphin in the amygdala, nucleus accumbens, and piriform cortex (regions important in stress and affect) in naïve WKY compared with SD rats (Carr et al., 2010; Dennis et al., 2016; Burke et al., 2019). The expression of the gene encoding for the MOP was higher in the hippocampus, but lower in the striatum, in the WKY rats compared with SD rats (Burke et al., 2019). WKY rats also have lower levels of NOP mRNA in the hippocampus, compared with SD rats (Burke et al., 2019). The functional/behavioural consequence of such alterations in the opioid system underlying the phenotype of WKY rats is yet to be determined. In addition, WKY rats demonstrate hyporesponsivity to opioid drugs including morphine, buprenorphine, butarphenol, and nalbuphine in tests of acute thermal (heat) pain, suggesting that a dysfunction of the opioid system may underlie the WKY phenotype (Hoffmann et al., 1998; Plesan et al., 1999; Terner et al., 2003; Avsaroglu et al., 2007; Hestehave et al., 2019b). WKY rats are also less sensitive to morphine in the test of mechanical hyperalgesia (Randall Selitto) in a model of chronic inflammatory pain, compared to SD rats (Hestehave et al., 2019b). Thus, the WKY rat represents a valuable model to explore the role of the opioid system in hyperalgesia associated (or co-occurring) with stress and negative affective states, which is a primary focus of the body of work presented in this thesis.

Table 1.4: Summary of studies investigating the role of the endogenous opioid system in hyperalgesia associated with stress and/or negative affect in animal models. ↑: increase, ↓: decrease, ↔: no change.

Target	Species/ strain	Stressor/pain model	Tests of pain and/or affect	Drugs used	Key behavioural findings	Changes in opioid system/method used	Reference
Pharmacological studies							
	ICR mice	Repeated cold water swim stress	Tail flick		Tolerance in the production of antinociception	↑ MOP and KOP and ↓ DOP in the brainstem, midbrain, and spinal cord (radioligand binding)	(Kim et al., 2003)
	SD rats	Chronic sleep disturbance	von Frey, Hargreaves', cold plate		SIH	↓ MOP and KOP in the spinal cord and dorsal root ganglia (Western blot)	(Wang et al., 2015c)
	SD rats	Neuropathic pain	von Frey, cold plate, sucrose preference		Mechanical and cold allodynia; anhedonia	Reduced opioid receptor availability in the insular cortex and striatum (PET imaging)	(Thompson et al., 2018)
	C57BL/6 mice	Neuropathic and inflammatory pain	von Frey, Hargreaves', Light-dark, elevated plus maze		Mechanical allodynia, thermal hyperalgesia; anxiety-like behaviour	↓ MOP and DOP and ↑ KOP activity in the amygdala and ↓ DOP activity in the frontal cortex (selective agonist-induced [^{35}S]GTPγS binding assay)	(Narita et al., 2006b, 2006a)
MOP/ KOP/ DOP	SD rats	Novel environment stress	Paw pressure vocalization test	Naltrexone (s.c.)	↓ SIH		(Le Roy et al., 2011)
MOP/ KOP/ DOP	WKY and SHR rats	Prolonged restraint stress	Tail flick test	Naloxone (i.v.)	↓ SIH		(Huang and Shyu, 1987)

MOP/ KOP/ DOP	SD rats	Forced swim stress	Hot plate test	Naloxone (i.p.)	↓ SIH		(Suarez-Roca et al., 2006)
MOP	SD and Wistar rats	Chronic electrical foot-shock	Tail-flick, formalin		SIH	↓ MOP mRNA in the spinal cord and DRG (RT-qPCR)	(Hormozi et al., 2018, 2019)
MOP	SD rats	Neuropathic pain	von Frey, cold plate, sucrose preference		Mechanical and cold allodynia, anhedonia	↓ MOP in the insula and striatum (immunohistochemistry)	(Thompson et al., 2018)
MOP	C57BL/6J mice	Neuropathic pain	von Frey, Hargreaves', cold plate, elevated plus maze, tail suspension		Mechanical and cold allodynia, thermal hyperalgesia; anxiety- and depression-like behaviour	↓ MOP in the hippocampus and spinal cord (Western blot)	(Ferreira-Chamorro et al., 2018)
MOP	SD rats	Forced swim stress	Hot plate	Naloxonazine (i.p.)	↓ SIH		(Suarez-Roca et al., 2006)
MOP	Swiss mice	Cold temperature (4°C)	Abdominal writhing	Morphine (i.p)	Reduced antinociceptive effect		(Schlen and Bentley, 1980)
MOP	C57BL/6 mice	Intermittent cold stress	Hargreaves'	Morphine (s.c. or i.c.v.)	Reduced antinociceptive effect		(Nishiyori et al., 2010)
				Morphine (i.t. or i.pl.)	↓ SIH		
MOP	C57BL/6 mice	Repeated restraint stress	Hot plate	Morphine (i.p.)	Reduced antinociceptive effect		(Puglisi-Allegra et al., 1986)
MOP	Wistar rats	Chronic restraint stress	Tail flick test	Morphine (i.p.)	Reduced antinociceptive effect		(da Silva Torres et al., 2003)

MOP	Wistar rats	Chronic restraint stress	Formalin (injected into the TMJ)	Morphine (i.p.)	Reduced antinociceptive effect		(Gameiro et al., 2005, 2006)
MOP	SD rats	Intermittent cold-water swim stress	Tail flick	Morphine	Reduced antinociceptive effect		(Girardot and Holloway, 1984)
MOP	SD rats	Forced swim stress	Hot plate	Morphine (i.p.)	Reduced antinociceptive effect		(Suarez-Roca et al., 2006)
MOP	Long-Evans hooded rats	Maternal separation	Tail flick, hot plate	Morphine (s.c.)	Reduced antinociceptive effect		(Kalinichev et al., 2001)
MOP	C57BL/6J mice	Intermittent cold stress	von Frey, Hargreaves'	Morphine (i.c.v)	Reduced antinociceptive effect		(Mukae et al., 2015)
MOP	ddY mice	Intermittent cold stress	Tail pressure	DAMGO (i.c.v.)	Reduced antinociceptive effect		(Omiya et al., 2000)
MOP	BALB/c mice	Unpredictable chronic mild stress	Hot plate	Morphine (i.p.)	Reduced antinociceptive effect of morphine, but not tramadol, in stressed rats		(Ide et al., 2015)
				Tramadol (i.p.)			
MOP	Wistar Han rats	Neuropathic pain	von Frey, elevated plus maze, forced swim	Tramadol (i.p.)	Reversed mechanical allodynia and associated anxiety- and depressive-like behavior		(Caspani et al., 2014)
MOP	SD rats	Repeated whisker pad stimulation	von Frey	Lesioning the RVM	Absence of SIH in rats with selective ablation of MOP neurons in the RVM		(Reynolds et al., 2011)

KOP	Hooded rats	Restraint stress	Paw pressure	MR1452	↑ SIH		(Pilcher and Browne, 1983)
KOP	SD rats	Forced swim stress	Hot plate	Nor-BNI (i.p.)	↔ SIH		(Suarez-Roca et al., 2006)
KOP	Wistar rats	SART stress	Randall-Selitto	SA14867 (p.o.)	↓ SIH		(Tsukahara-Ohsumi et al., 2011)
KOP	ddY mice	Intermittent cold stress	Tail pressure	U50488 (s.c. or i.t.)	Greater antinociceptive effect in stressed mice than controls		(Omiya et al., 2000)
DOP	SD rats	Repeated intermittent foot shock	Tail flick		SIH	↓ Met- and Leu-enkephalin in the brain (high pressure liquid chromatography)	(McGivern et al., 1983)
DOP	SD rats	Forced swim stress	Hot plate	Naltrindole (i.p.)	↔ SIH		(Suarez-Roca et al., 2006)
DOP	ddY mice	Intermittent cold stress	Tail pressure	DPDPE	↔ SIH		(Omiya et al., 2000)
DOP	C57BL/6J mice	Neuropathic and inflammatory pain	von Frey, Hargreaves', cold plate, tail suspension	UFP-512 (i.p)	Reversed mechanical and cold allodynia and thermal hyperalgesia and associated depression-like behaviour		(Polo et al., 2019)
NOP	SD rats	Single-prolonged stress	von Frey, Hargreaves'		SIH	↑ N/OFQ levels in CSF and serum (radioimmunoassay)	(Zhang et al., 2012)
NOP	Wistar rats	Partial restraint stress	Colorectal distension	N/OFQ peptide (i.p.)	↓ SIH		(Agostini et al., 2009)
				N/OFQ peptide (i.c.v)	↔ SIH		

				UFP-101 (i.p.)	↑ SIH		
NOP	SD rats	Single-prolonged stress	von Frey, Hargreaves'	JTC-801 (i.p.)	↓ SIH	• ↓ N/OFQ levels in serum, CSF, PAG, and hippocampus (radioimmunoassay) • ↓ NOP mRNA levels in the amygdala and PAG (RT-qPCR) • ↓ NOP in the PAG (Western blot)	(Zhang et al., 2015)
Genetic studies							
KOP, PDYN	KOP-/- and PDYN-/- mice (C57BL/6 background)	Monosodium iodoacetate model of knee osteoarthritis	von Frey, sucrose preference, elevated plus maze, novel object recognition		Greater mechanical allodynia and anhedonia but reduced anxiety-like behaviour and cognitive deficit in knockout mice compared with wildtype counterparts		(Negrete et al., 2017)
	WKY rats					↑ KOP mRNA in the LC of Wistar-Kyoto versus Sprague-Dawley rats (gene microarray and RT-qPCR)	(Pearson et al., 2006)
	WKY rats					• ↑ MOP mRNA in the hippocampus (RT-qPCR) • ↓ MOP mRNA in the striatum (RT-qPCR)	(Carr et al., 2010; Dennis et al., 2016; Burke et al., 2019)

						• ↑ KOP mRNA in the nucleus accumbens, amygdala, and hippocampus (RT-qPCR) • ↑ KOP in the piriform cortex (Western blot) • ↑ dynorphin in the nucleus accumbens (ELISA) • ↓ NOP mRNA in the hippocampus	
	WKY rats		Hot plate, warm water tail withdrawal	Morphine (s.c., i.p., or i.v.), buprenorphine (i.p. or i.v.), butorphanol (i.p.) and nalbuphine (i.p. or i.v.)	Reduced antinociceptive effect in the WKY strain		(Hoffmann et al., 1998; Plesan et al., 1999; Terner et al., 2003; Avsaroglu et al., 2007; Hestehave et al., 2019b)
	WKY rats	Inflammatory pain (CFA-induced)	Randall-Selitto	Morphine (s.c.)	Reduced antinociceptive effect in the WKY strain		(Hestehave et al., 2019b)

1.4.7. Endogenous opioid system and cognition

Given the abundant expression of the opioid receptors in corticolimbic areas of the brain, it is not surprising that they have been implicated in cognitive processes such as learning and memory. These cognitive domains are often impaired in chronic pain or affective disorders (Moriarty et al., 2011; Jacobson et al., 2018) and, hence, the opioid system may play a role in comorbidity of chronic pain with affective disorders and impaired cognition. Early studies in healthy subjects showed that agonists of MOP (Saarialho-Kere et al., 1989; Walker et al., 2001) and KOP (MacLean et al., 2013) impaired normal cognitive processes, including recall for episodic and spatial memory (Martín del Campo et al., 1992). Preclinical studies in knockout transgenic mice have revealed insights into the role of opioid receptors in learning and memory. MOP null mice exhibit impairment in spatial memory in two distinct tasks, the 8-arm radial and Morris water maze (Jamot et al., 2003; Jang et al., 2003), possibly due to reduced hippocampal long-term potentiation (Matthies et al., 2000), which is a long lasting and activity-dependent synaptic plasticity important for memory formation (Bliss and Collingridge, 1993). No spatial memory impairment is seen in KOP knockout mice (Jamot et al., 2003). Genetic deletion of PDYN has no effect on spatial and recognition memory in aged mice compared to wild-type littermates (Ménard et al., 2013). Interestingly, pharmacological activation of KOP in PDYN knockout mice impairs recognition memory (Bilkei-Gorzo et al., 2014). Constitutive knockout of DOP results in impairment in object location learning and short-term memory (Le Merrer et al., 2013), as well as deficiencies in place conditioning tasks (Le Merrer et al., 2011). In addition, deletion of NOP gene improves spatial memory in Morris water maze task (Manabe et al., 1998) Overall, these genetic studies suggest that all four opioid receptors have a role in learning and memory, with MOP and DOP facilitating, and KOP and NOP impeding, these cognitive processes.

Pharmacological manipulation of opioid receptors also reveals some interesting results. Spatial learning in the water maze task is impaired by blocking MOP (Meilandt et al., 2004) or activating KOP (Daumas et al., 2007) in the hippocampus in rodents. Also, the KOP selective antagonist nor-BNI reverses dynorphin B-induced or stress-induced impairment in spatial (Sandin et al., 1998) and recognition (Carey et al., 2009) memory in rodents. DOP has been studied the most with respect to cognitive processing. However, pharmacologically activating DOP either facilitates or impairs learning depending on the task employed to assess memory performance (Pellissier et al., 2018), warranting further

investigation to understand the role of DOP in cognitive performance. Nevertheless, the DOP antagonist naltrindole reduces performance in the hippocampus-dependent object-place recognition task in mice (Le Merrer et al., 2013). With regard to NOP system, NOP agonists impair memory whereas NOP antagonists have been shown to block this effect (Andero, 2015). Taken together, the data from pharmacological studies are largely in line with those obtained from genetic studies and further support role of the opioid system in memory. However, only two studies to date have investigated the opioid system with respect to comorbidity of chronic pain with negative affect and cognitive impairment. Deletion of the KOP gene in a mouse model of knee osteoarthritis pain enhances mechanical allodynia and pain-induced anhedonia but reduces anxiety-like behaviour and deficits in object recognition memory (Negrete et al., 2017). In another study, using the rat model of knee osteoarthritis pain, Grégoire et al showed that although low doses of morphine administered systemically or into the BLA did not reduce mechanical allodynia, pain-induced anxiety-like behaviours and impairment in spatial memory were reversed (Grégoire et al., 2014). These studies indicate a complex receptor-dependent role of the opioid system in modulating pain and associated negative affect and cognitive impairment.

1.5. Overall hypothesis and aims of the thesis

The studies discussed above provide substantial evidence for a key role of the opioid system in the interrelationship between pain and stress-related negative affective states (anxiety and depression). However, the influence of genetic background on such interaction remains largely unexplored. In addition, the underlying neural substrates within the descending pain pathway mediating this enhanced nociceptive response are still poorly understood. Moreover, only a few studies so far have investigated the involvement of the opioid system in comorbidity between chronic pain and negative affect and/or cognitive impairment. Thus, the overarching hypothesis of the work presented herein is that alteration in the expression and/or functionality of the endogenous opioid system in key components of the descending pain pathway may underpin hyperalgesia to inflammatory pain in WKY rats, compared with SD counterparts. In addition, given the hyperalgesic and stress hyperresponsive phenotype of the WKY rats, we hypothesised that this strain will have exacerbated response/deficits in behavioural domains relating to nociceptive response, negative affect, and cognition in a model of chronic inflammatory pain.

Our overall objective was to advance our understanding of the role of the opioid system in pain-negative affect interactions. In this respect, the aims of the studies discussed in this body of work were as follows:

- To investigate the effects of pharmacologically modulating MOP on acute thermal (heat) and formalin-evoked nociceptive behaviour and anxiety- and depression-related behaviour in SD and WKY rats (Chapter 2).
- To examine the role of MOP in the ventrolateral subcolumn of the PAG on formalin-evoked nociceptive behaviour in SD and WKY rats (Chapter 3).
- To investigate the effects of pharmacologically modulating the KOP on acute thermal (heat) and formalin-evoked nociceptive behaviour, the affective component of persistent inflammatory pain, and anxiety- and depression-related behaviour in SD and WKY rats (Chapter 4).
- To investigate nociceptive hypersensitivity and associated deficits in affect and cognition in a model of chronic inflammatory pain in WKY and SD rats (Chapter 5).
- To examine the effects of pharmacologically modulating KOP and DOP on nociceptive hypersensitivity and associated deficits in affect and cognition in a model of chronic inflammatory pain in SD rats (Chapter 6).

2. Chapter 2: Effects of modulating mu-opioid receptors on nociceptive and anxiety/depression-related behaviours in Wistar-Kyoto and Sprague-Dawley rats

2.1. Introduction

Pain and negative affective states, such as anxiety and depression, share a complex reciprocal relationship. Numerous clinical studies have reported a high prevalence of chronic pain disorders (Bair et al., 2003; Asmundson and Katz, 2009; Wiech and Tracey, 2009) and alterations in the sensitivity to, and tolerance of, pain (Lautenbacher et al., 1999; Kain et al., 2000; Rhudy and Meagher, 2000; Bar et al., 2005) in patients with anxiety and depression. On the other hand, the prevalence rates of anxiety or depression in chronic pain patients can reach as high as 77% or 80%, respectively (Poole et al., 2009; Knaster et al., 2012). Moreover, patients with chronic pain are at greater risk of developing anxiety or major depressive disorder when compared to the general population (McWilliams et al., 2003; Twillman, 2008). Such comorbid conditions have significant clinical implications, often amplifying the severity of each individual disorder and resulting in poor therapeutic outcomes and reduced quality of life of the patients. Thus, co-occurrence of pain and negative affect is more debilitating than either condition alone and imposes a huge socioeconomic burden.

In preclinical studies, animal models have been used to investigate neurobiological mechanisms underlying pain-negative affect interactions (for review see Liu and Chen, 2014; Li, 2015). One such preclinical model is the inbred Wistar-Kyoto (WKY) rat strain, a genetic model with a number of anxiety and depression-related deficits (Gentsch et al., 1987; Paré and Redei, 1993; Paré, 1994; Malkesman and Weller, 2009; Burke et al., 2010). WKY rats display hyperresponsivity to stress (Tejani-Butt et al., 1994; Rittenhouse et al., 2002; De La Garza and Mahoney, 2004), including increased susceptibility to stress-induced ulcers (Paré, 1989a; Redei et al., 1994) and heightened activation of the stress-responsive neuroendocrine pathways (Redei et al., 1994; Rittenhouse et al., 2002; De La Garza and Mahoney, 2004). In addition, WKY rats exhibit a hyperalgesic phenotype. WKY rats show thermal hyperalgesia in the hot plate test compared to Sprague-Dawley (SD) rats (Burke et al., 2010), a commonly used control

comparator strain. Greater nociceptive responding following intraplantar formalin injection (noxious inflammatory stimulus) has also been reported in the WKY rats, compared to SD counterparts (Burke et al., 2010; Rea et al., 2014). Several studies have demonstrated visceral hypersensitivity in the WKY rats (Gunter et al., 2002; Gibney et al., 2010; O'Mahony et al., 2010; O' Mahony et al., 2013). Moreover, WKY rats exhibit mechanical allodynia under baseline conditions (Taylor et al., 2001; Hestehave et al., 2019a) and following peripheral nerve injury (Zeng et al., 2008). Thus, the WKY rat is a valuable experimental model for studying altered nociceptive responding in the context of comorbid pain and anxiety/depression and the influence of genetic background thereon.

The endogenous opioid system plays a key role in the regulation of various physiological functions including pain, emotion, and response to stress (Benarroch, 2012a; Lutz and Kieffer, 2013; Valentino and Van Bockstaele, 2015). A growing body of evidence has also indicated a maladaptive response of the opioid system in comorbid pain and affective disorders (for review see Ferdousi and Finn, 2018). Emerging research in the WKY rat model suggests that the mu-opioid (MOP) system is dysfunctional in this strain, which may account for the characteristic behavioural phenotype. Recently, Burke and coworkers (Burke et al., 2019) reported higher expression of the gene encoding the MOP in the hippocampus (a key brain region for processing mood) in WKY versus SD rats. Pharmacological studies have revealed a differential effect of opioidergic drugs targeting the MOP in WKY rats. Buprenorphine, a partial MOP agonist and a functional KOP antagonist, reduced immobility in the forced swim test in WKY rats; this antidepressant-like effect of buprenorphine was not seen in SD rats (Browne et al., 2015; Burke et al., 2019). In the tail withdrawal test for noxious heat stimulus, buprenorphine did not affect latency to respond in the WKY rats (Avsaroglu et al., 2007). Furthermore, WKY rats exhibit reduced responsivity to the antinociceptive effects of morphine, a high efficacy MOP agonist, in tests of acute heat nociception (Hoffmann et al., 1998; Plesan et al., 1999; Terner et al., 2003; Hestehave et al., 2019b), suggesting that a dysregulation of the MOP may underlie the WKY phenotype. However, to date, morphine sensitivity in the WKY rats has not been investigated in other persistent or chronic pain models, except for a recent study by Hestehave and colleagues who has reported reduced morphine sensitivity in a rat model of chronic inflammatory pain (Hestehave et al., 2019b). Additionally, potential underlying neurobiological differences in the MOP system that

may underpin such strain-related differences in nociception and morphine sensitivity in the WKY rats have not yet been investigated.

Therefore, this chapter examines the hypothesis that reduced expression and/or functionality of MOP may underlie hyperalgesia and differential responsivity to MOP agonism in WKY rats, compared to SD counterparts. More specifically, the aims of the experiments described in this chapter were as follows:

- To compare the effects of systemic administration of the MOP agonist morphine on nociceptive responding to acute (thermal) and persistent (tonic inflammatory) noxious stimuli in WKY versus SD rats.
- To compare the effects of systemic administration of morphine on anxiety- and depression-related behaviours in WKY versus SD rats.
- To further elucidate whether these behavioural responses of morphine were mediated via MOP by employing a highly selective MOP antagonist cyprodime (Schmidhammer et al., 1989) in tests of nociception and negative affect in WKY and SD rats.
- To assess if the observed behavioural differences between WKY and SD rats were due to a difference in the pharmacokinetics of morphine in the two strains.
- To compare the expression of the genes encoding MOP and POMC (prepropeptide precursor to the endogenous ligand β-endorphin) in discrete brain regions involved in processing pain and negative affect in WKY and SD rats.

2.2. Materials and methods

2.2.1. Animals

Experiments were carried out in male WKY and SD rats (8-10 weeks old, 170-230 g on arrival; Envigo, Bicester, UK). Upon arrival, all animals were housed in groups of 3-4 per cage in plastic bottom cages (45x20x20 cm^3) containing 3Rs basic bedding (>99% recycled paper; 3Rs Lab, Fibrecycle Ltd, North Lincolnshire, UK) and sizzle nest material (LBS Biotechnology, Horley, UK) to acclimatise to the unit. Animals were housed singly in cages for 7 days prior to commencing behavioural testing. A separate cohort of naïve WKY and SD rats were pair-housed according to strain throughout the study and did not undergo any behavioural testing. The animals were maintained under a standard 12:12 h light/dark cycle (lights on from 07:00-19:00 h) in a temperature- (21±2°C) and humidity-controlled (45-55%) room. All experimental procedures were carried out during the light phase between 08:00 and 18:00 h. Food (14% protein rodent diet; Envigo, Bicester, UK) and water were available *ad libitum*. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway. The work was carried out under license from the Health Products Regulatory Authority in the Republic of Ireland and in accordance with EU Directive 2010/63. The study was also designed and carried out in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

2.2.2. Chemicals and drug preparation

The MOP agonist morphine sulphate (10 mg/mL solution for injection; MercuryPharma, Surrey, UK) was purchased from a local pharmacy in Galway, Ireland. The MOP antagonist cyprodime hydrochloride was obtained from Tocris (Bristol, UK). Both morphine sulphate and cyprodime hydrochloride were dissolved in the vehicle citrate buffer (50 mM citric acid monohydrate in sterile deionised water, pH 5; Lennox, Dublin, Ireland) and administered via the subcutaneous (s.c.) route in an injection volume of 1 mg/mL. The final doses of the drugs were corrected for the salt form and given as mg free base equivalent per kg body weight. All drugs were freshly prepared on the days of behavioural testing. For Experiment 3, formalin (Sigma-Aldrich, Wicklow, Ireland) was diluted from a stock of 37% to 2.5% in sterile 0.89% w/v NaCl (Fisher Scientific, Leicestershire, UK) on the test day.

2.2.3. Study design

The study consisted of three separate experiments (Figure 2.1). Animals were randomly assigned to treatment groups in Experiment 1 and subsequently reallocated into treatment groups for Experiment 2 and 3 following a within-subjects modified Latin square design to ensure counterbalancing of treatments over the course of the study (see Table 2.1 for experimental groups and n numbers). In Experiments 2 and 3, each animal received two injections to control for the times of drug administration. The sequence of treatments was randomised in all experiments to control for the order of testing and time of the day. A period of 7 days was allowed for washout of the drugs between each experiment.

Experiment 1 compared dose response of morphine (0.5, 2, 5, and 7.5 mg/kg, s.c.) on nociceptive responding to noxious heat and anxiety-/depression-related behaviours in WKY and SD rats. Following morphine/vehicle administration, rats were tested on the hot plate (at +30 and +60 min), followed 5 min later by exposure to the elevated plus maze (EPM) for 5 min, then in the open field (OF) for another 5 min and subsequently in the forced swim test (FST) for a period of 15 min. Doses of morphine used in subsequent experiments were chosen based on the results of Experiment 1. In Experiment 2, the effects of morphine (2 mg/kg, s.c.) and cyprodime (0.3 and 1 mg/kg, s.c.), alone or in combination, on thermal nociceptive responding and anxiety-/depression-related behaviours were investigated in WKY versus SD rats in a series of behavioural tests as in Experiment 1. Cyprodime was administered subcutaneously 30 min before morphine injection. The dose and time point of cyprodime administration were determined from a pilot study (data not shown) conducted in the facility of Alkermes Inc demonstrating the timecourse of its action in blocking the antinociceptive effect of morphine in the hot plate test. Experiment 3 assessed the effects of morphine (0.5 and 2 mg/kg, s.c.) and cyprodime (0.3 mg/kg, s.c.), alone or in combination, on formalin-evoked nociceptive behaviour in WKY and SD rats. Cyprodime was administered 15 min before morphine injection. 30 min following morphine administration, all rats received an intraplantar formalin injection into the right hindpaw under brief isoflurane anaesthesia. The rats were assessed in the formalin test for 60 min after which they were immediately euthanised by decapitation and trunk blood was collected. The naïve animals were also euthanised by decapitation, whole brain was harvested, and brain regions of interest were dissected out. All tissues were collected in RNase-free Eppendorf tubes, and snap frozen on dry ice before storing at -80°C for later use.

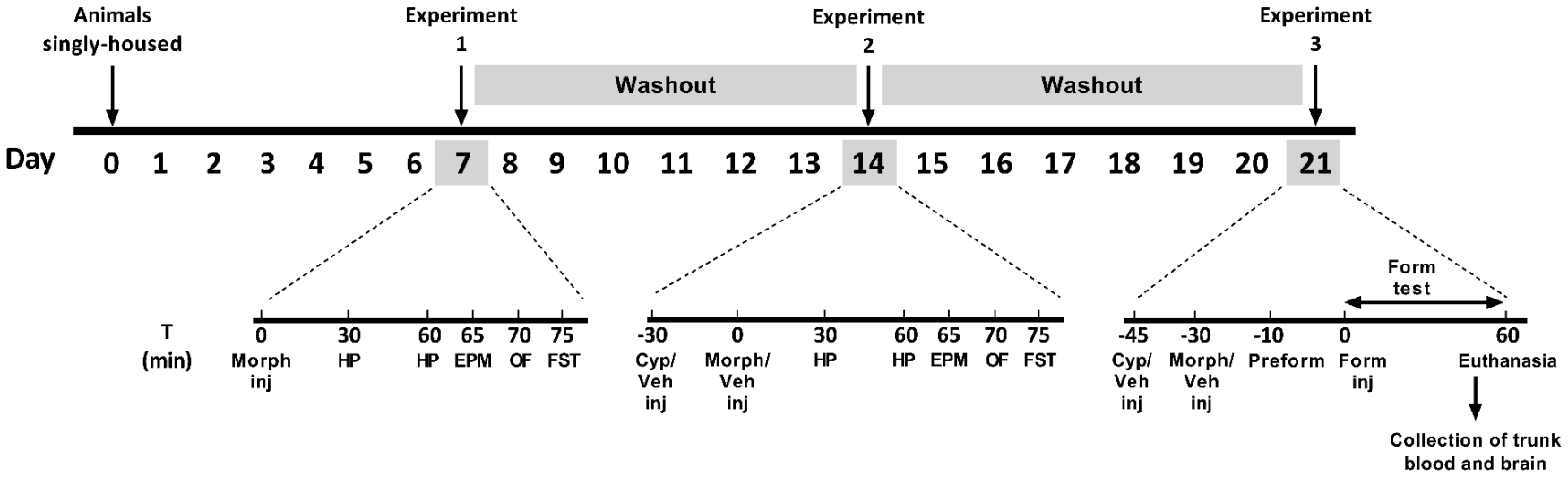


Figure 2.1: Schematic of the study outlining the design, timeline and behavioural tests of Experiments 1, 2, and 3. A period of 7 days was allowed for washout of the drugs between each experiment. Cyp: cyprodime, EPM: elevated plus maze, Form: formalin, FST: forced swim test, HP: hot plate, OF: open field, Morph: morphine, Preform: pre-formalin, T: time, Veh: vehicle.

Table 2.1: Summary of experimental groups for Experiments 1-3. n: number, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Group	Strain	Treatment, s.c.	n
Experiment 1			
1	SD	Vehicle	11
2	SD	Morphine 0.5 mg/kg	11
3	SD	Morphine 2 mg/kg	11
4	SD	Morphine 5 mg/kg	11
5	SD	Morphine 5 mg/kg	11
6	WKY	Vehicle	11
7	WKY	Morphine 0.5 mg/kg	11
8	WKY	Morphine 2 mg/kg	11
9	WKY	Morphine 5 mg/kg	11
10	WKY	Morphine 5 mg/kg	11
Experiment 2			
1	SD	Vehicle	11
2	SD	Morphine 2 mg/kg	11
3	SD	Morphine 2 mg/kg + Cyprodime 0.3 mg/kg	11
4	SD	Morphine 2 mg/kg + Cyprodime 1 mg/kg	11
5	SD	Cyprodime 1 mg/kg	11
6	WKY	Vehicle	11
7	WKY	Morphine 2 mg/kg	11
8	WKY	Morphine 2 mg/kg + Cyprodime 0.3 mg/kg	11
9	WKY	Morphine 2 mg/kg + Cyprodime 1 mg/kg	11
10	WKY	Cyprodime 1 mg/kg	11
Experiment 3			
1	SD	Vehicle	9
2	SD	Morphine 0.5 mg/kg	9
3	SD	Morphine 2 mg/kg	9
4	SD	Morphine 0.5 mg/kg + Cyprodime 0.3 mg/kg	9
5	SD	Morphine 2 mg/kg + Cyprodime 0.3 mg/kg	10
6	SD	Cyprodime 0.3 mg/kg	9
7	WKY	Vehicle	9
8	WKY	Morphine 0.5 mg/kg	9
9	WKY	Morphine 2 mg/kg	9
10	WKY	Morphine 0.5 mg/kg + Cyprodime 0.3 mg/kg	9
11	WKY	Morphine 2 mg/kg + Cyprodime 0.3 mg/kg	10
12	WKY	Cyprodime 0.3 mg/kg	9

2.2.4. Behavioural testing

2.2.4.1. Nociceptive responding

2.2.4.1.1. Hot plate test

The hot plate test was used to assess nociceptive responding to an acute (phasic) noxious thermal stimulus. On the test day (in Experiments 1 and 2), baselines were measured once in WKY and SD rats 30 min before administering morphine/vehicle. Following drug injection, rats were tested on the hot plate at two time points (+30 and +60 min). The animal was placed onto the hot plate (IITC Life Science Inc, CA, USA) that was heated at 54.5±1°C. Nociceptive behaviour was measured manually with a stopwatch as the time elapsed (latency to respond in seconds) between placing the animal on the hot plate and when the animal first licked one of its hindpaws or jumped off the surface of the hot plate. A cut-off point of 40 s was set to avoid any tissue damage. The hot plate was cleaned between animals using a cleaning solution (Milton:tap water = 1:5; Milton Sterilising Fluid, CW Distributors Ltd, Galway, Ireland).

2.2.4.1.2. Formalin-evoked nociceptive responding

In Experiment 3, nociceptive responding to a persistent (tonic) noxious inflammatory stimulus was assessed in WKY and SD rats in the formalin test as described previously (Burke et al., 2010; Rea et al., 2014; Jennings et al., 2016; Okine et al., 2017). On the test day, rats were habituated in a darkened Perspex arena with clear base (30x30x40 cm^3, 30 lux) for 10 min prior to formalin injection. This is called the pre-formalin trial. To assess any effect of the drug on locomotor activity, the general exploratory behaviours (distance moved in the arena, rearing, and grooming) of the rats were recorded using a video camera located underneath the arena during pre-formalin trial. The rats then received an intraplantar formalin injection (2.5%, 50 µL) into the right hindpaw under brief isoflurane anaesthesia (3% in 0.8 L/min O_2) and were immediately returned to the same Perspex arena. The test began when the rat righted itself lasting for 60 min and nociceptive and exploratory behaviours were recorded. All behaviours were analysed later with the aid of EthoVision video tracking software (EthoVision XT, version 11.5; Noldus, Wageningen, Netherlands).

Formalin-evoked nociceptive behaviour was categorised as times spent (a) elevating the formalin-injected paw above the floor without contact to any other surface (P1) and (b) holding, licking, biting, shaking, or flinching the injected paw (P2). A weighted

composite pain score [CPS = (P1+2*P2)/(total duration of analysis period)] was then obtained as described previously (Watson et al., 1997). Exploratory behaviours in pre- and post-formalin trials were also rated. The diameter of the formalin-injected hindpaw was measured using Vernier callipers immediately before injecting formalin and following euthanasia as a measure formalin-induced oedema. The number of faecal pellets produced during pre- and post-formalin trials was also counted. The arena was cleaned between animals with Milton cleaning solution.

2.2.4.2. Anxiety- and depression-related tests

2.2.4.2.1. Elevated plus maze test

The EPM was employed to assess the effects of drug treatment on anxiety-related behaviours in WKY and SD rats. The arena was elevated 50 cm above the floor of the testing room and consisted of a white plus-shaped wooden maze with two arms enclosed by walls (30 cm high, closed arms) and two open arms with no enclosure. The floor of the maze was covered in a black rubbery material. Each arm was 50 cm in length and 10 cm in width. A central platform (10x10 cm^2) connected the four arms. The light levels were fixed at 60 lux in the open arms and 25 lux in the closed arms. A video camera was positioned over the arena to record the behaviours of the animal. On the test day, 5 min after the hot plate test, rats were removed from the home cage, placed in the centre zone of the maze with their heads facing an open arm, and the behaviours were recorded for 5 min that were later analysed using EthoVision system. Entries and duration in open arms over the 5 min period were used as experimental indices for anxiety-like behaviour (Walf and Frye, 2007). Entry into an arm was counted when all four paws of the rat were on that arm. The EPM was cleaned between animals with Milton cleaning solution.

2.2.4.2.2. Open field test

Following EPM test, rats were immediately exposed to the OF test. The circular arena consisted of a white floor (plastic covered wood flooring) of diameter 75 cm and reflective aluminium wall 40 cm high. Animals were placed in the centre of the novel open environment that was brightly lit (280-300 lux). A video camera positioned 35 cm above the floor of the OF arena was used to record the behaviours for 5 min for subsequent analysis using the EthoVision system. Entries and duration in the centre zone (diameter 45 cm) were assessed as indices for anxiety-related behaviour. Other behaviours measured included distance moved in the arena, latency to leave centre zone, and number

of pellets produced (defaecation) over the 5 min trial period. The OF was cleaned between animals with Milton cleaning solution.

2.2.4.2.3. Forced swim test

The effects of drug treatment on behavioural despair in WKY and SD rats were assessed using a single exposure of the modified FST (Porsolt et al., 2001; Slattery and Cryan, 2012). It should be noted that the WKY rats exhibit spontaneous immobility in the FST and, hence, it is deemed that a pretest exposure to forced swimming 24 h before may not be necessary to induce helplessness or behavioural despair in this strain (Getachew et al., 2008; Tizabi et al., 2012; Akinfiresoye and Tizabi, 2013). Immediately after the OF test, rats were placed into an inescapable glass cylinder (45 cm high and 20 cm in diameter) containing water (depth: 30 cm) at 25±1°C for 15 min. Behaviours were recorded using a video camera positioned in front of the cylinder. Ethovision software was used to score rat behaviours that included time spent immobile (only floating, no additional movement of any paw), swimming (horizontal movements), and climbing (vigorous, upward directed movements). After the FST, rats were towel-dried and placed in a cage, which was on a heating pad, for 15 min before returning to home cage.

2.2.5. Tissue harvesting

2.2.5.1. Blood

Immediately after decapitation of the animals, trunk blood was collected in 10 mL EDTA-coated tubes (Becton Dickinson, Plymouth, UK) to prevent coagulation and centrifuged at 1600g (Eppendorf Centrifuge 5810 R, Hamburg, Germany) for 10 min at 4°C. Clear straw-coloured plasma was collected in microcentrifuge tubes and stored at -80°C until further use.

2.2.5.2. Whole brain

Following decapitation, the skin on top of the head was cut with scissors from the neck until the eyes to expose the skull. The optic ridge between the eyes was broken with rongeurs. The tip of a small scissors was inserted into the foramen magnum and pressure exerted to break the posterior part of the cranium. Then the small scissors were used to carefully cut the cranium starting from the upper edge of the foramen magnum and moving along the midline, always maintaining pressure upward and outward to preserve the brain tissue underneath. The occipital, parietal, and frontal parts of cranium were

removed to expose the brain. After removing the dura mater, forceps with curved tips was carefully slid between the bone and the lateral part of one of the brain hemispheres. The process was repeated for the other brain hemisphere, carefully removing the brain from the skull. The olfactory, optical, and trigeminal nerve attachments were gently cut with the forceps to aid the process. Once removed from the skull, regions of interest (in the following order: hypothalamus, PFC, amygdala, hippocampus, and PAG) were rapidly dissected on ice using forceps and a scalpel blade fitted on a handle. Gross dissected regions were weighed (Table 2.2) and collected in RNase-free Eppendorf tubes, snap frozen on dry ice, and stored at -80°C for subsequent use.

2.2.6. Liquid chromatography/tandem mass spectrometry

Morphine levels in plasma were measured in the vehicle- and morphine-treated (0.5 and 2 mg/kg) WKY and SD rats to investigate whether strain-related pharmacokinetic differences may underpin the observed difference in antinociceptive effectiveness of morphine in the two strains. The plasma samples were analysed by our industry collaborator Alkermes Inc in their facility. A method was optimised for measuring the level of morphine in rat plasma. All reagents were purchased from J. T. Baker® (NJ, USA). Samples were analysed using a 20 µL aliquot volume and a protein-precipitation extraction procedure was employed. A mixture of plasma and acetonitrile (1:4) was vortexed and centrifuged. The supernatant was then removed and diluted with water (1:1). 5 µL of this mixture was injected for liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis. The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) and was delivered via Shimadzu LC-30AD at a flow rate of 0.4 mL/min onto an Acquity UPLC BEH C18 column (particle size: 1.7 µm, length: 50 mm, internal diameter: 2 mm; Waters®, MA, USA). Under these conditions, the retention time for elution of morphine was 1.97 min. An AB Sciex Qtrap® 5500 was operated in the Selected Reaction Monitoring (SRM) mode under optimized conditions for the detection of morphine and morphine-d3 (internal standard) positive ions formed by electrospray ionization. Morphine concentrations were calculated with a 1/x linear regression over a concentration range of 1 to 1000 ng/mL using the internal standard morphine-d3.

2.2.7. Real-time quantitative polymerase chain reaction (RT-qPCR)

Quantitative real-time polymerase chain reaction (RT-qPCR) was employed to analyse the expression of the relevant genes in gross dissected brain tissues from naïve animals. Total RNA was extracted from the tissues (mean weights given in Table 2.2) using the Macherey-Nagel NucleoSpin® RNA extraction kit (Nucleospin RNA, Fisher Scientific, Dublin, Ireland) according to manufacturer instructions. Tissue was homogenised in 353.5 µL of lysis buffer (RA1) containing β-mercaptoethanol (Sigma-Aldrich, Wicklow, Ireland) for 3-5 s using an automated homogenizer (Ultra-Turrax® tissue disruptor, Staufen, Germany). The tissue homogenate was kept on ice until transferred to a Nucleospin filter with violet ring and centrifuged at 11000g for 1 min to reduce viscosity and clear the lysate. The lysate was then treated with 350 µL of 70% molecular grade ethanol (Sigma-Aldrich, Wicklow, Ireland), transferred to a Nucleospin RNA column with light blue ring and centrifuged at 11000g for 30 s to bind the RNA to the membrane. The membrane column was then desalted by adding 350 µL membrane desalting buffer (MDB) and centrifuging again at 11000g for 1 min to dry the membrane. The samples were incubated with 10 µL rDNase for 15 min at room temperature to degrade any DNA. The samples were then serially washed using washing buffers (200 µL RAW2, 600 µL RA3, and 250 µL RA3). Finally, RNA was eluted in 50 µL of RNase-free water (Sigma-Aldrich, Wicklow, Ireland) and collected in 1.5 mL RNase-free microcentrifuge tubes. The concentration, purity, and integrity of RNA extracted were measured using a Nanodrop spectrophotometer (ND-1000 Nanodrop, Labtech International, Ringmer, UK). RNA concentration was determined by measuring optical density (OD) at 260 nm. RNA integrity and purity were determined by measuring the ratios OD260/OD280 and OD260/OD230, respectively, where a ratio of approximately 1.8-2.0 was deemed indicative of good quality and pure RNA. All RNA samples were within the acceptable range for both integrity and purity. Samples were then equalised to the same concentration of RNA for each region using RNase-free water (Table 2.2) and stored at -80°C until required for cDNA synthesis.

Table 2.2: Weight of the tissue after dissection, concentration, quality, and purity of isolated RNA, and equalised RNA concentration of the brain regions studied. Data are expressed as mean ± SEM. Amyg: amygdala, Hipp: hippocampus, Hypo: hypothalamus, PAG: periaqueductal grey, PFC: prefrontal cortex.

Tissue	Weight (mg)	Mean RNA concentration (ng/µL)	Integrity (260:280)	Purity (260:230)	Equalised RNA concentration (ng/µL)
PFC	79.52±4.14	204.02±9.75	2.1-2.2	1.8-2.1	150
Amyg	71.66±4.94	167.41±9.83	1.9-2.1	1.8-2.0	75
Hipp	53.51±3.20	212.51±15.24	2.1-2.2	1.9-2.1	80
Hypo	26.69±1.76	292.22±22.48	2.1-2.2	1.9-2.1	130
PAG	62.43±2.37	106.06±1.64	2.1-2.2	1.7-2.1	70

Equal amount of total RNA from each sample was reverse transcribed into cDNA. All reagents for cDNA synthesis were purchased from Bio-Sciences (Dublin, Ireland). Two master mixes were made up as shown below in Table 2.3. 10 µL equalised RNA from each sample was added to a newly labelled PCR tube where 2 µL of master mix 1 was added to each tube. The mixture was then heated to 65ºC for 5 min in a thermocycler (PTC-200 Thermal Cycler, MJ Research, MN, USA) followed by quick chilling on ice. The contents of the tube were collected by brief centrifugation. 7 µL master mix 2 was then added to each tube and incubated at 37ºC for 2 min on the thermocycler. 1 µL superscript III reverse transcriptase was then added to each sample and mixed gently. Samples were incubated at room temperature for 10 min and loaded again on the thermocycler to incubate further at 50ºC for 50 min. The reaction was inactivated by heating the samples at 70ºC for another 15 min. Finally, the newly synthesised cDNA samples were diluted (1:4) using RNase-free water and stored at -20ºC.

Table 2.3: Composition of master mixes for cDNA synthesis.

Master mix	Reagents	Volume per sample (µL)
Master mix 1	Random Primers (250 ng/ µL)	1
	10mM dNTP mix	1
	Total volume (µL)	**2**
Master mix 2	5X First Strand Buffer	4
	0.1M DTT	2
	RNase Out	1
	Total volume (µL)	**7**

cDNA strands were then analysed by RT-qPCR using the Applied Biosystems StepOne Plus™ Real-Time PCR System (Bio-Sciences, Dublin, Ireland). TaqMan gene expression assays (Bio-Sciences, Dublin, Ireland) containing forward and reverse primers and FAM-labelled TaqMan MGB probes were used (Bio-Sciences, Dublin, Ireland) to quantify the genes of interest. The assay IDs and fluorescent labels for the rat genes examined are listed in Table 2.4. VIC-labelled β-actin was used as the endogenous control (house-keeping) gene. All samples were run in a multiplex assay. Briefly, 2.5 µL cDNA samples were pipetted in duplicate into an optical 96 well plate (Bio-Sciences, Dublin, Ireland). 7.5 µL reaction mixture (containing 5 µL Taqman Universal PCR master mix, 0.5 µL target (*Oprm1*/*Pomc*) primer, 0.5 µL endogenous control primer, and 1.5 µL RNase-free water) was then added to the cDNA samples to give a total reaction volume of 10 µL per well. No template control was included in all assays, where the reaction mixture was added to the wells but the cDNA was replaced with RNase-free water. Plates were then sealed with transparent adhesive covers, spun at 1000g for 1 min to ensure complete mixing, and ran in StepOnePlus™ Real Time PCR machine (Bio-Sciences, Dublin, Ireland). The cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of (95°C for 15 s followed by 60°C for 1 min). Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems 7500 System SDS Software 1.3.1 and exported to Microsoft Excel for final analysis. Ct values of the target gene were normalised to the endogenous control gene expression for each sample. ΔΔCt method was employed to calculate the relative expression of the target gene to the control gene for each sample

and data were expressed as a percentage of the mean of the ΔΔCt for the control group (SD naïve).

Table 2.4: Assay IDs and fluorescent labels for the target and endogenous control genes examined in rat brain tissues by RT-qPCR.

Target gene	Assay ID	Fluorescent label	Control gene	Assay ID	Fluorescent label
MOP (*Oprm1)*	Rn01430371_m1	FAM	β-actin (*Actb*)	Rn00667869_m1	VIC
POMC (*Pomc)*	Rn00595020_m1	FAM			

2.2.8. Statistical analysis

The SPSS statistical software (IBM SPSS Statistics, version 24 for Windows; SPSS Inc, Chicago, IL, USA) was used to analyse all data. In all datasets the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was considered an outlier and excluded from subsequent analysis. The normality and homogeneity of variance of the datasets were checked using Shapiro-Wilk and Levene's tests, respectively. The timecourse behavioural data were analysed with repeated measures analysis of variance (RM ANOVA) with strain and drug treatment as between-subject factors and time as within-subject factor. For RM ANOVA, sphericity of the datasets was checked with Mauchly's Test for Sphericity; if the assumption of sphericity was violated, a Greenhouse-Geisser correction was used. All other behavioural data except for defaecation were analysed using two-way ANOVA with strain and drug treatment as factors. *Post hoc* Student-Newman-Keuls (SNK) test was carried out for pairwise group comparisons, where appropriate. MOP and POMC gene expression data in naïve rats were analysed using Student's unpaired, two-tailed t-test. The level of significance was deemed $p<0.05$. If the data were not normally distributed and/or the variance was not homogeneous, three transformations were applied, in the order: square root, natural logarithm, and ranking of the data values to evaluate if parametric statistics can be used. In addition, if the highest standard deviation was less than or equal to 2 times

the smallest standard deviation for the particular dataset being analysed, parametric statistical approach was still employed (Moore et al., 2009).

If any dataset was ordinal (e.g. defaecation) or found to be not normally distributed and/or the variance was not homogeneous even after transformation, non-parametric statistical analysis was performed using Kruskal-Wallis (KW) one-way analysis of variance by rank, followed by *post hoc* Mann-Whitney U-test (*p*-value was adjusted using Bonferroni-Holm correction for multiple comparisons), where appropriate. In all cases statistical analysis of a dataset was carried out for all the groups together. However, in some instances, the groups are graphed separately to present the results clearly in a reader-friendly format. Data are expressed as either mean ± standard error of the mean (SEM) or median with interquartile range (IQR) depending on the statistical approach undertaken, parametric or non-parametric, respectively.

2.3. Results

2.3.1. Experiment 1

2.3.1.1. Effects of morphine on nociceptive responding to noxious heat stimulus in WKY and SD rats

The hot plate test was used to assess the dose response of morphine (0.5-7.5 mg/kg, s.c.) on nociceptive responding to acute noxious heat in WKY and SD rats (Figure 2.2). At baseline, latency to respond on the hot plate did not differ between WKY and SD rats. Animals were tested on the hot plate again at +30 and +60 min post morphine administration. Two-way RM ANOVA revealed significant effects of time ($F_{1.675,162.477}$=47.217, ***p*<0.001**), time x strain ($F_{1.675,162.477}$=17.990, ***p*<0.001**), and time x treatment ($F_{6.700,162.477}$=9.901, ***p*<0.001**) in tests of within-subjects effects. A significant overall effect of treatment ($F_{4,97}$=32.807, ***p*<0.001**) was also found in tests of between-subjects effects on response latency. Further *post hoc* SNK analysis showed that morphine dose-dependently increased response latency in both SD (Figure 2.2A) and WKY rats (Figure 2.2B), compared to their respective vehicle counterparts. However, the minimum effective doses for morphine-induced antinociception were 0.5 and 2 mg/kg in SD and WKY rats, respectively, indicating a reduced responsivity to low dose morphine in the WKY versus SD rats in the hot plate test.

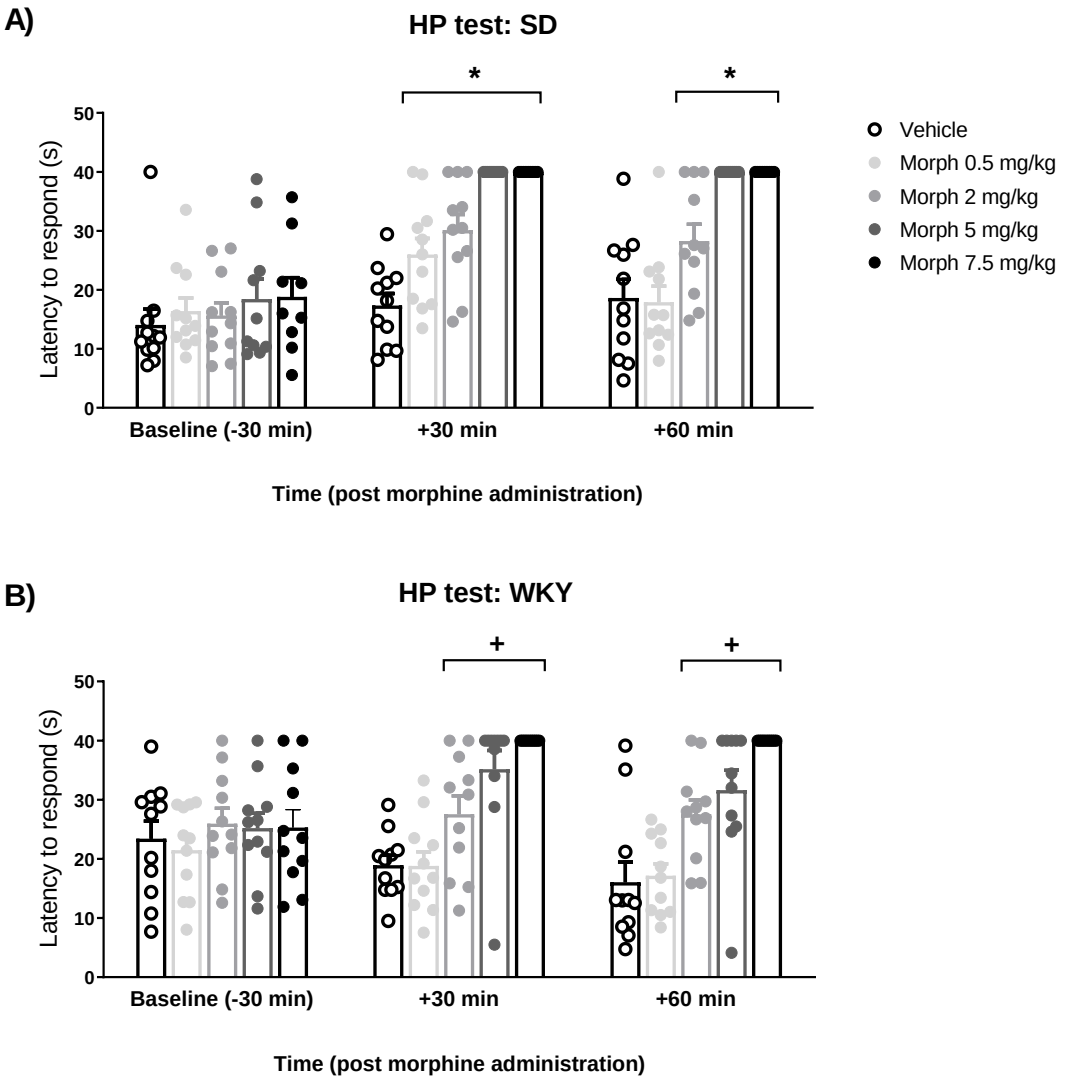


Figure 2.2: Effects of morphine (0.5-7.5 mg/kg, s.c.) on nociceptive responding to acute noxious heat stimulus (Experiment 1) in (A) SD and (B) WKY rats. Data are expressed as mean ± SEM, n = 9-11/group. *p<0.05 (vs SD-Vehicle), ^{+}p<0.05 (vs WKY-Vehicle). HP: hot plate, Morph: morphine, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

2.3.1.2. Effects of morphine on anxiety-related behaviour and general locomotor activity in WKY and SD rats

After the hot plate test, animals were exposed to the EPM and then immediately to the OF to evaluate the effects of morphine on anxiety-related behaviours and general locomotor activity. In the EPM, an increase in open arm activity (time spent and/or number of entries) reflects reduced anxiety-related behaviour. KW test revealed a significant main effect ($\chi^2(9)=17.211$, $\mathbf{p<0.05}$) on time spent in open arms; further *post hoc* pairwise comparisons revealed there were no significant differences between drug-treated groups within each strain or between the two strains (Figure 2.3A). KW also showed a significant main effect ($\chi^2(9)=38.550$, $\mathbf{p<0.001}$) on number of entries into open arms. *Post hoc* test revealed that morphine at dose 7.5 mg/kg significantly reduced entries into open arms in SD (SD-Morphine 7.5 mg/kg vs SD-Vehicle, $\mathbf{p<0.001}$), but not in WKY, rats (Figure 2.3B). Activity in closed arms (time spent and/or number of entries) in the EPM provides an indirect measure of spontaneous locomotor activity. A significant main effect ($\chi^2(9)=23.000$, $\mathbf{p<0.01}$) on time spent in closed arms was revealed in KW test but *post hoc* analysis showed there were no significant difference between relevant groups (Figure 2.3C). KW test also revealed a significant main effect ($\chi^2(9)=72.570$, $\mathbf{p<0.001}$) on number of entries into closed arms. Further *post hoc* test showed vehicle-treated WKY rats exhibited a significant reduction in closed arm entries, compared to SD counterparts (WKY-Vehicle vs SD-Vehicle, $\mathbf{p<0.001}$) (Figure 2.3D), indicating their hypolocomotor trait. Morphine 7.5 mg/kg significantly reduced closed arm entries in both SD and WKY rats (SD-Morphine 7.5 mg/kg vs SD-Vehicle, $\mathbf{p<0.001}$; WKY-Morphine 7.5 mg/kg vs WKY-Vehicle, $\mathbf{p<0.001}$) (Figure 2.3D), suggesting a locomotor impairing effect of the high dose morphine.

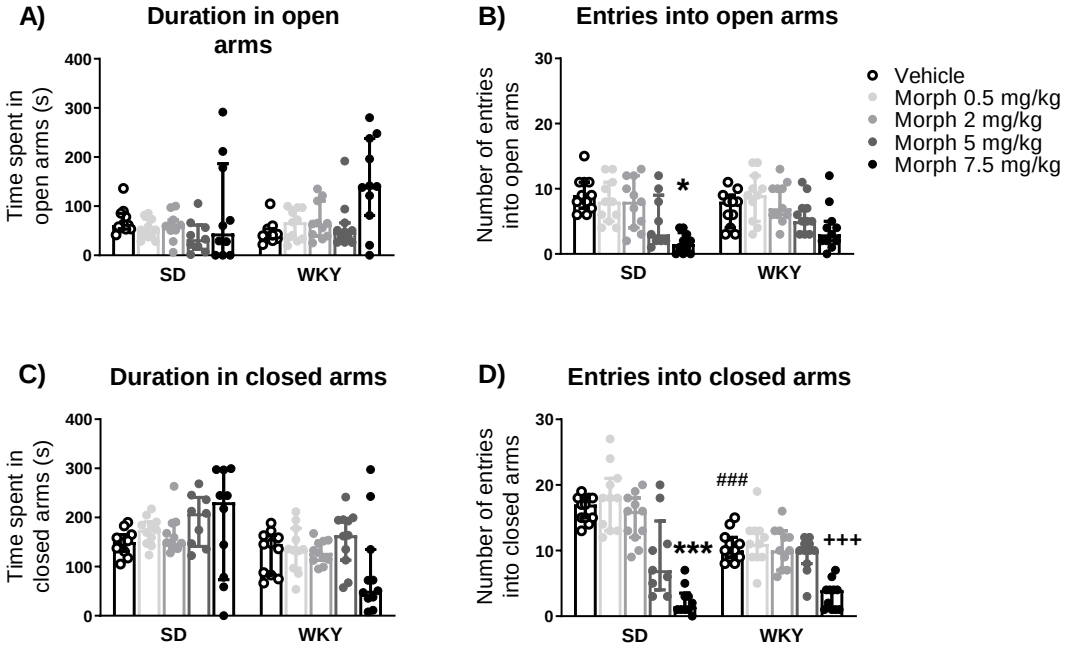


Figure 2.3: Effects of morphine (0.5-7.5 mg/kg, s.c.) on duration and number of entries in (A and B) open and (C and D) closed arms in the EPM test in SD and WKY rats (Experiment 1). Data are expressed as median with IQR, n = 9-11/group. *p<0.05, ***p<0.001 (SD-Morphine 7.5 mg/kg vs SD-Vehicle), ^{+++}p<0.001 (WKY-Morphine 7.5 mg/kg vs WKY-Vehicle), $^{###}p$<0.001 (WKY vs SD counterpart). EPM: elevated plus maze, Morph: morphine, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

In the OF test, KW test revealed a significant main effect ($\chi^2(9)=69.150$, ***p*<0.001**) on the distance moved in the OF arena (Figure 2.4A). Vehicle-treated WKY rats exhibited a significant reduction in distance moved in the OF compared to SD controls (WKY-Vehicle vs SD-Vehicle, ***p*<0.001**), indicating their hypolocomotor phenotype. High doses of morphine significantly reduced distance moved in the arena in the SD rats (SD-Morphine 5 mg/kg vs SD-Vehicle, ***p*<0.01**; SD-Morphine 7.5 mg/kg vs SD-Vehicle, ***p*<0.001**), whereas distance moved was increased in the WKY counterparts (WKY-Morphine 5 mg/kg or WKY-Morphine 7.5 mg/kg vs WKY-Vehicle, ***p*<0.01**), suggesting a differential effect of morphine on locomotor activity in WKY versus SD rats.

Entries and time spent in the centre zone of the arena are used as indices to measure anxiety-like behaviours in rats. KW revealed a significant main effect ($\chi^2(9)=28.356$, ***p*<0.01**) on number of entries into the centre zone of the arena (Figure 2.4C). *Post hoc* test revealed that vehicle-treated WKY rats demonstrated a significant reduction in centre zone entries compared to SD counterparts (WKY-Vehicle vs SD-Vehicle, ***p*<0.01**). Morphine reduced entries into the centre zone in SD rats with the dose 5 mg/kg reaching statistical significance compared to vehicle controls (SD-Morphine 5 mg/kg vs SD-Vehicle, ***p*<0.01**); these effects were not observed in the WKY rats. KW also revealed a significant main effect ($\chi^2(9)=20.652$, ***p*<0.05**) on time spent in the centre zone (Figure 2.4D). Subsequent *post hoc* test indicated there were no significant difference between relevant groups within or between strains. Faecal output, another measure for anxiogenic behaviour (O'Malley et al., 2010; Hyland et al., 2015), during the 5 min trial period in the OF was also recorded (Figure 2.4B). KW analysis revealed a significant main effect ($\chi^2(9)=72.056$, ***p*<0.01**) on pellets excreted. *Post hoc* test showed that the number of faecal pellets excreted by vehicle-treated WKY rats was significantly higher than SD counterparts (WKY-Vehicle vs SD-Vehicle, ***p*<0.01**). Of note, morphine significantly reduced faecal output in the WKY, but not in the SD, rats. KW test also revealed a significant main effect ($\chi^2(9)=28.179$, ***p*<0.01**) on latency to leave the centre zone (Figure 2.4E). *Post hoc* analysis showed that the vehicle-treated WKY rats exhibited higher latency to initially leave the centre of the OF at the beginning of the trial, compared to SD counterparts (WKY-Vehicle vs SD-Vehicle, ***p*<0.001**).

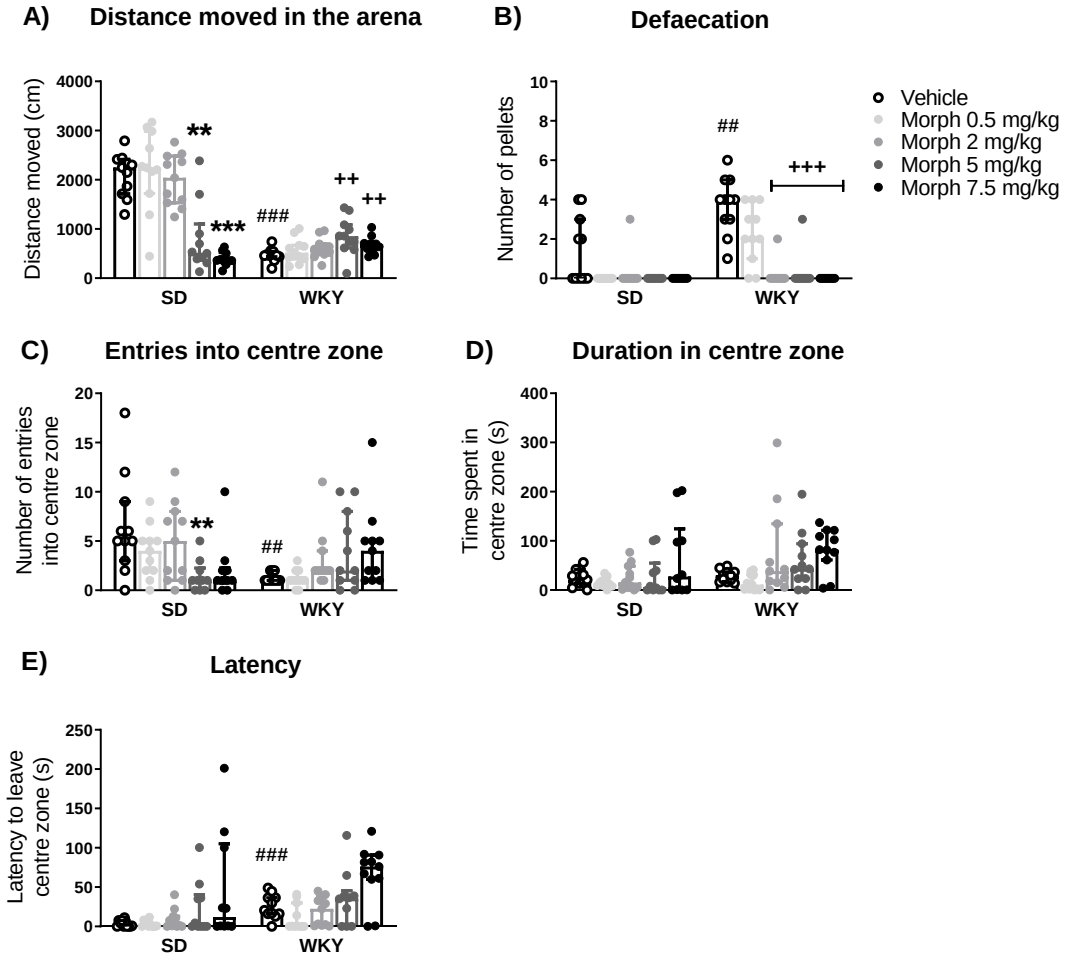


Figure 2.4: Effects of morphine (0.5-7.5 mg/kg, s.c.) on (A) distance moved in the arena, (B) defaecation (number of pellets produced), (C) number of entries into centre zone, (D) time spent in centre zone, and (E) latency to leave centre zone in the OF test in SD and WKY rats (Experiment 1). Data are expressed as median with IQR, n = 9-11/group. $^{**}p<0.01$, $^{***}p<0.001$ (SD-Morphine 5 mg/kg or SD-Morphine 7.5 mg/kg vs SD-Vehicle); $^{++}p<0.01$, $^{+++}p<0.001$ (WKY-Morphine 2 mg/kg or WKY-Morphine 5 mg/kg or WKY-Morphine 7.5 mg/kg vs WKY-Vehicle); $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ (WKY vs SD counterpart). OF: open field, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

2.3.1.3. Effects of morphine on behaviours in the forced swim test in WKY and SD rats

Following OF test, animals underwent a single 15 min exposure to FST to evaluate the effects of morphine in this test of behavioural despair (Figure 2.5). Both passive (immobility) and active (swimming and climbing) behaviours were rated and analysed. Two-way ANOVA revealed significant effects of strain ($F_{4,107}$=18.814, ***p*<0.001**) and treatment ($F_{1,107}$=7.666, ***p*<0.001**) but no strain x treatment interaction ($F_{4,107}$=1.908, p>0.05) on time spent immobile in the FST. Further *post hoc* test indicated there was no significant difference in duration of immobility in vehicle-treated WKY versus SD rats. However, morphine 7.5 mg/kg markedly increased duration of immobility in the WKY rats compared to vehicle controls (WKY-Morphine 7.5 mg/kg vs WKY-Vehicle, ***p*<0.05**); this effect was not observed in the SD counterparts (Figure 2.5A). The data was further analysed as temporal profile divided into 5 min time bins (Table 2.5). RM ANOVA revealed significant effects of time ($F_{1.278,125.287}$=23.836, ***p*<0.001**) and time x treatment interaction ($F_{5.114,125.287}$=15.212, ***p*<0.001**) in tests of within-subjects effects and a significant overall effect of strain ($F_{1,98}$=24.255, ***p*<0.001**), treatment ($F_{4,98}$=10.303, ***p*<0.001**), and strain x treatment interaction ($F_{4,98}$=3.980, ***p*<0.01**) in tests of between-subjects effects on duration of immobility. *Post hoc* tests showed that vehicle-treated WKY rats exhibited progressive increase in duration of immobility, compared to SD controls, that reached statistical significance in the last 5 min of the trial ($T_{10\text{-}15}$: WKY-Vehicle vs SD-Vehicle, ***p*<0.05**), indicating a depressive-like phenotype in the WKY rats. High doses of morphine (5 and 7.5 mg/kg) significantly increased time spent immobile in both strains ($T_{0\text{-}5}$: SD-Morphine 7.5 mg/kg vs SD-vehicle, ***p*<0.05**; WKY-Morphine 5 mg/kg or WKY-Morphine 7.5 mg/kg vs WKY-Vehicle, ***p*<0.05**) in the first 5 min of the trial, which reduced to the level of control rats over the course of the trial.

Two-way ANOVA revealed significant effects of treatment ($F_{4,107}$=11.235, ***p*<0.001**) and strain x treatment interaction ($F_{4,107}$=4.182, ***p*<0.01**), but not of strain ($F_{1,107}$=0.261, p>0.05), on time spent swimming during 15 min trial. *Post hoc* test showed that morphine increased duration of swimming in both SD (SD-Morphine 2 mg/kg or SD-Morphine 5 mg/kg or SD-Morphine 7.5 mg/kg vs SD-Vehicle, ***p*<0.05**) and WKY (WKY-Morphine 2 mg/kg or WKY-Morphine 5 mg/kg vs WKY-Vehicle, ***p*<0.05**) rats. In addition, a significant effect of treatment ($F_{4,107}$=11.700, ***p*<0.001**) only was observed on time spent climbing during the 15 min trial. Further *post hoc* testing revealed that morphine

significantly reduced duration of climbing in the WKY rats (WKY-Morphine 2 mg/kg or WKY-Morphine 5 mg/kg or WKY-Morphine 7.5 mg/kg vs WKY-Vehicle, ***p*<0.05**).

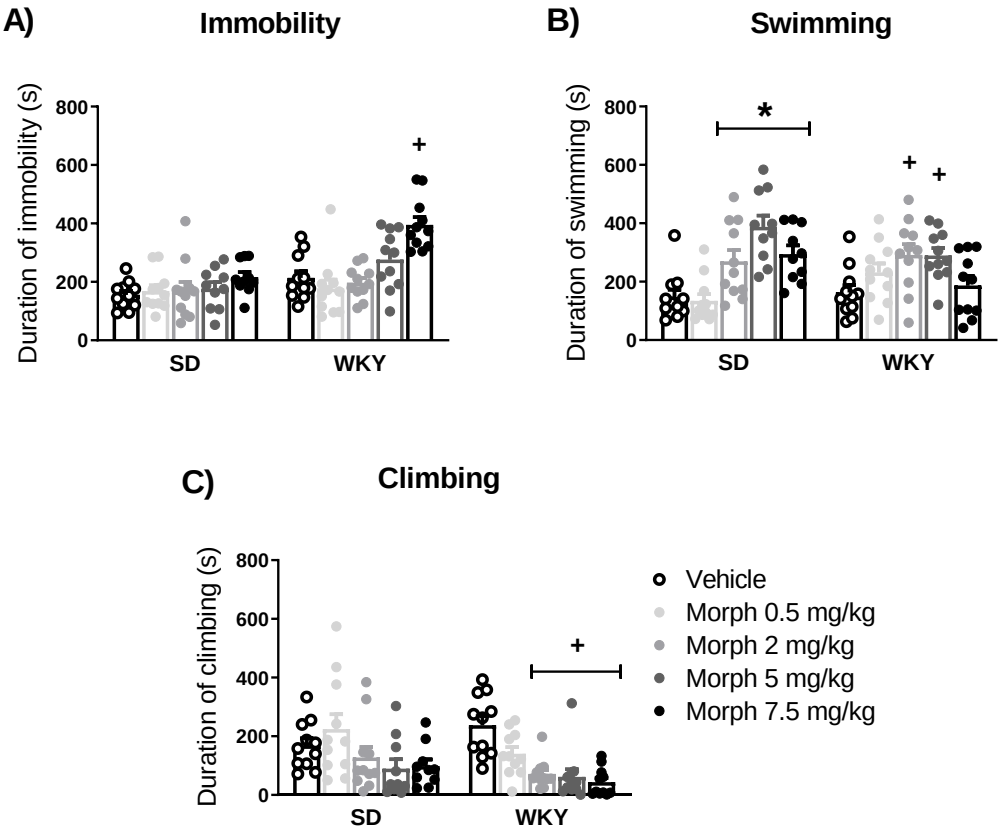


Figure 2.5: Effects of morphine (0.5-7.5 mg/kg, s.c.) on duration of (A) immobility, (B) swimming, and (C) climbing over 15 min in the FST in SD and WKY rats (Experiment 1). Data are expressed as mean ± SEM, n = 10-11/group. **p*<0.05 (SD-Morphine 2 mg/kg or SD-Morphine 5 mg/kg or SD-Morphine 7.5 mg/kg vs SD-Vehicle), ^{+}p<0.05 (WKY-Morphine 2 mg/kg or WKY-Morphine 5 mg/kg or WKY-Morphine 7.5 mg/kg vs WKY-Vehicle). FST: forced swim test, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Table 2.5: Temporal profile of the effect of morphine (0.5-7.5 mg/kg, s.c.) on duration of immobility in the FST in SD and WKY rats (Experiment 1). Data are presented in 5 min time bins and expressed as mean ± SEM, n = 10-11/group. *p<0.05 (SD-Morphine 7.5 mg/kg vs SD-Vehicle), ^{+}p<0.05 (WKY-Morphine 5 mg/kg or WKY-Morphine 7.5 mg/kg vs WKY-Vehicle), $^{\#}p$<0.05 (WKY vs SD counterpart). FST: forced swim test, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Groups	**Time spent (s)**		
	0-5 min	**5-10 min**	**10-15 min**
SD-Vehicle	40.52±7.38	51.10±5.80	63.75±4.96
SD-Morph 0.5 mg/kg	55.29±10.20	51.30±5.70	62.03±7.54
SD-Morph 2 mg/kg	75.56±22.71	41.29±7.52	52.68±5.62
SD-Morph 5 mg/kg	79.23±11.25	34.20±7.23	63.29±7.62
SD-Morph 7.5 mg/kg	**131.66±24.62***	35.44±7.00	48.52±6.65
WKY-Vehicle	39.92±7.42	73.76±9.05	**100.04±11.12**$^{\#}$
WKY-Morph 0.5 mg/kg	58.23±21.33	49.22±6.09	69.33±7.84
WKY-Morph 2 mg/kg	66.36±6.87	55.21±7.44	75.71±9.80
WKY-Morph 5 mg/kg	**114.33±14.93**$^{+}$	78.07±10.51	83.86±9.29
WKY-Morph 7.5 mg/kg	**191.22±15.95**$^{+}$	93.31±8.16	110.49±10.37

2.3.2. Experiment 2

2.3.2.1. Effects of morphine and cyprodime, alone or in combination, on nociceptive responding to noxious heat stimulus in WKY and SD rats

After a week of washout and reallocation of rats to new treatment groups according to a Latin square design, Experiment 2 was carried out. WKY and SD rats were injected via the subcutaneous route with morphine (2 mg/kg) and cyprodime (0.3 or 1 mg/kg), alone or in combination, and nociceptive responding to noxious heat stimulus was evaluated in the hot plate test (Figure 2.6). There was no difference in the baseline response latency of the WKY and SD rats, similar to Experiment 1. Two-way RM ANOVA revealed significant effects of time ($F_{2,194}$=5.465, ***p*<0.01**), time x strain ($F_{2,194}$=5.099, ***p*<0.01**), and time x treatment ($F_{8,194}$=5.273, ***p*<0.001**) in tests of within-subjects effects and a significant overall effect of treatment ($F_{4,97}$=3.375, ***p*<0.05**) in tests of between-subjects effects on response latency. *Post hoc* tests revealed that systemic administration of morphine 2 mg/kg significantly increased latency to respond on the hot plate in SD rats (SD-Morphine 2 mg/kg vs SD-Vehicle, ***p*<0.05**; Figure 2.6A), but not in the WKY counterparts (WKY-Morphine 2 mg/kg vs WKY-Vehicle, $p>0.05$; Figure 2.6B), 60 min post-drug administration. Pretreatment with cyprodime (0.3 or 1 mg/kg) attenuated morphine-induced antinociception in the SD rats. Cyprodime (1 mg/kg), administered alone, did not affect response latency on the hot plate in either strain.

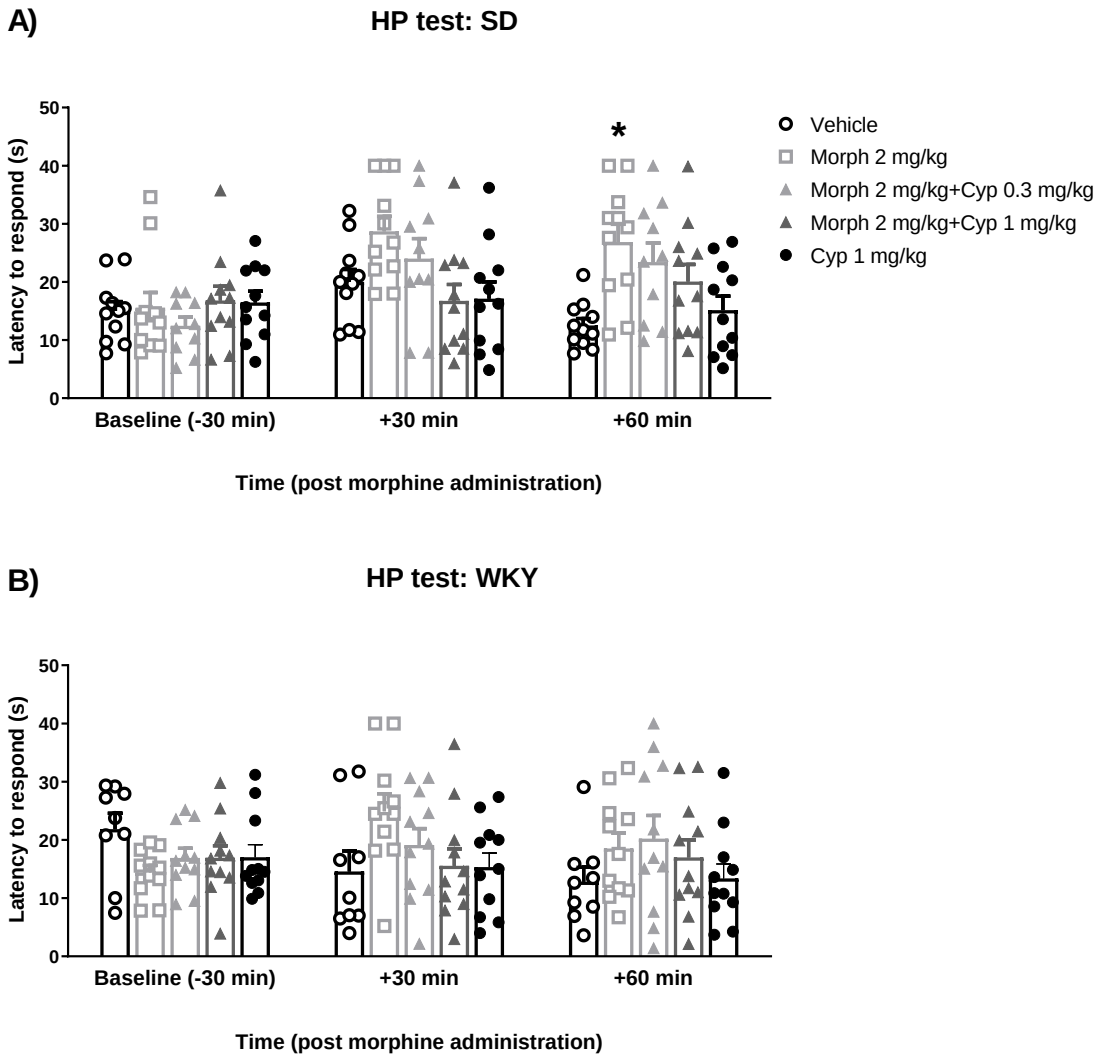


Figure 2.6: Effects of morphine (2 mg/kg, s.c.) and cyprodime (0.3 or 1 mg/kg, s.c.), alone or in combination, on nociceptive responding to acute noxious heat stimulus (Experiment 2) in (A) SD and (B) WKY rats. Data are expressed as mean ± SEM, n = 9-11/group. *p<0.05 (SD-Morphine 2 mg/kg vs SD-Vehicle). Cyp: Cyprodime, HP: hot plate, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

2.3.2.2. Effects of morphine and cyprodime, alone or in combination, on anxiety-related and general locomotor activity in WKY and SD rats

Following the hot plate assay, animals were tested in the EPM and thereafter in the OF. In the EPM, two-way ANOVA revealed there was no significant effect of strain, treatment, or strain x treatment interaction on activities in the open arms (Time spent in open arms- strain: $F_{1,107}$=1.821, p>0.05; treatment: $F_{4,107}$=0.808, p>0.05; strain x treatment: $F_{4,107}$=1.817, p>0.05. Entries into open arms- strain: $F_{1,107}$=2.474, p>0.05; treatment: $F_{4,107}$=1.118, p>0.05; strain x treatment: $F_{4,107}$=0.903, p>0.05) (Figure 2.7A and 2.7B). Similarly, no significant effects were found in the ANOVA on the time spent in closed arms (strain: $F_{1,107}$=0.982, p>0.05; treatment: $F_{4,107}$=0.644, p>0.05; strain x treatment: $F_{4,107}$=1.588, p>0.05) (Figure 2.7C). However, a significant effect of strain ($F_{1,107}$=39.445, $\mathbf{p<0.001}$), but not of treatment ($F_{4,107}$=0.407, p>0.05) or strain x treatment interaction ($F_{4,107}$=0.626, p>0.05), was observed on the number of entries into closed arms. Further *post hoc* SNK test found that vehicle-treated WKY rats exhibited significant reduction in closed arm entries compared to SD controls (WKY-Vehicle vs SD-Vehicle, $\mathbf{p<0.05}$), indicating their hypolocomotor trait (Figure 2.7D). Morphine and/or cyprodime at the doses tested did not affect entries into the closed arms in either strain.

Figure 2.7: Effects of morphine (2 mg/kg, s.c.) and cyprodime (0.3 or 1 mg/kg, s.c.), alone or in combination, on duration and number of entries in (A and B) open and (C and D) closed arms in the EPM test in SD and WKY rats (Experiment 2). Data are expressed as mean ± SEM, n = 10-11/group. a: effect of strain (p<0.001); $^{\#}p$<0.05 (WKY vs SD counterpart). Cyp: Cyprodime, EPM: elevated plus maze, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

In the OF test, KW analysis revealed a significant main effect ($\chi^2(9)=56.724$, ***p*<0.001**) on the distance moved in the OF arena (Figure 2.8A). Vehicle-treated WKY rats exhibited a significant reduction in distance moved in the OF compared to SD counterparts (WKY-Vehicle vs SD-Vehicle, ***p*<0.001**), indicating their hypolocomotor phenotype. Systemic administration of morphine and/or cyprodime did not affect distance moved in SD or WKY rats. KW analysis revealed significant main effects on pellets excreted during the 5 min trial ($\chi^2(9)=31.244$, ***p*<0.001**) (Figure 2.8B). *Post hoc* test showed there was no significant difference between relevant groups. There was no significant main effect ($\chi^2(9)=10.296$, $p>0.05$) in KW test on number of entries into the centre zone (Figure 2.8C). KW analysis revealed significant main effects on time spent in the centre zone ($\chi^2(9)=20.691$, ***p*<0.05**) (Figure 2.8D) and latency to leave centre of the arena

($\chi^2(9)=21.617$, ***p*<0.01**) (Figure 2.8E). However, no significant difference was found between relevant groups in the *post hoc* analysis.

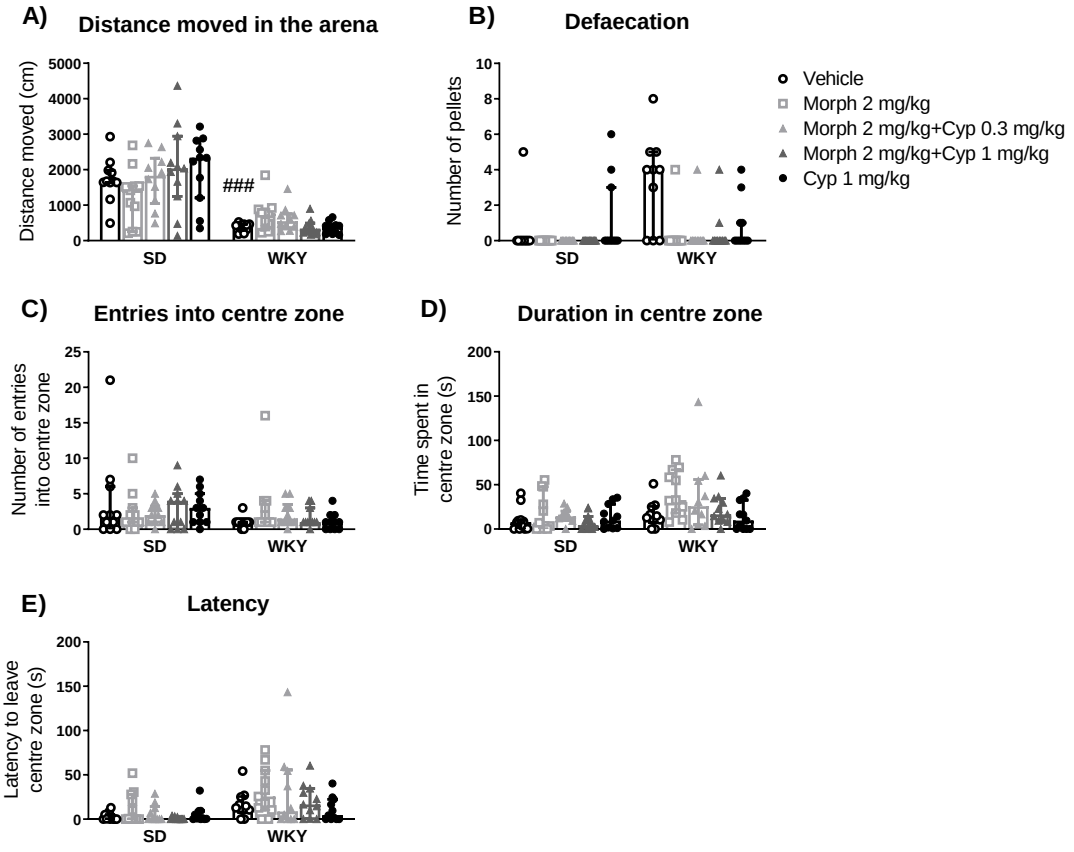


Figure 2.8: Effects of morphine (2 mg/kg, s.c.) and cyprodime (0.3 or 1 mg/kg, s.c.), alone or in combination, on (A) distance moved in the arena, (B) defaecation (number of pellets produced), (C) number of entries into centre zone, (D) time spent in centre zone, and (E) latency to leave centre zone in the OF test in SD and WKY rats (Experiment 2). Data are expressed as median with IQR, n = 10-11/group. $^{###}p<0.001$ (WKY vs SD counterpart). Cyp: Cyprodime, OF: open field, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

2.3.2.3. Effects of morphine and cyprodime, alone or in combination, on behaviours in the forced swim test in in WKY and SD rats

Immediately after the OF test, rats were exposed to another session of FST for 15 min (Figure 2.9). No significant effect ($\chi^2(9)=6.647$, $p>0.05$) on duration of immobility was observed in the KW test. KW revealed significant main effects on duration of swimming ($\chi^2(9)=33.694$, ***p*<0.001**) and climbing ($\chi^2(9)=30.866$, ***p*<0.001**) but further *post hoc* analysis showed there were no significant differences between relevant groups.

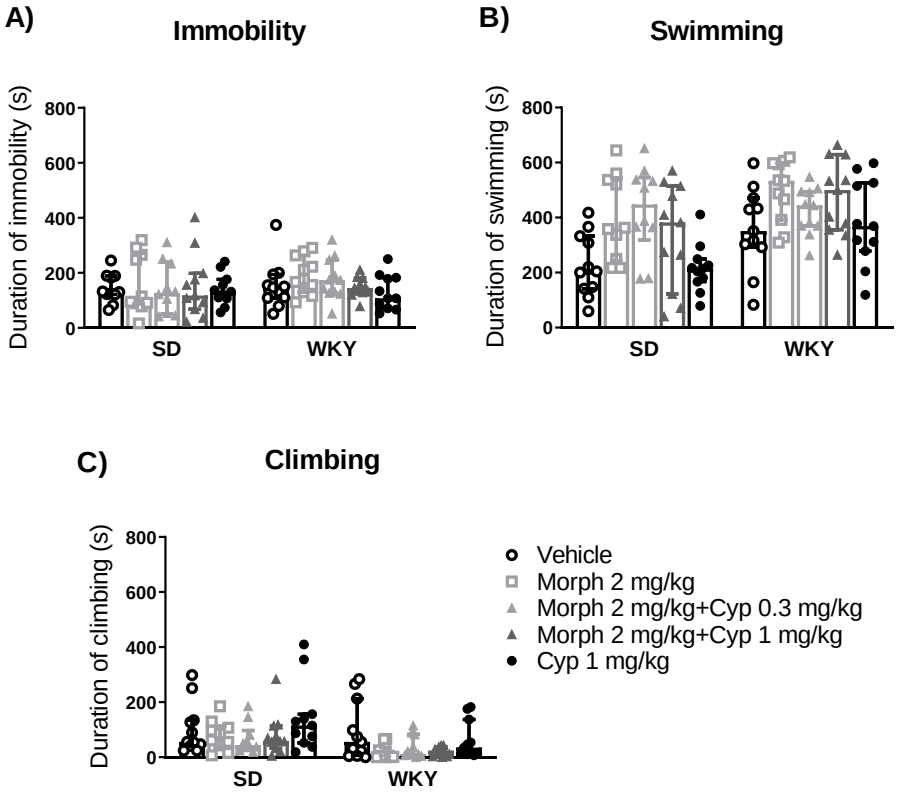


Figure 2.9: Effects of morphine (2 mg/kg, s.c.) and cyprodime (0.3 or 1 mg/kg, s.c.), alone or in combination, on duration of (A) immobility, (B) swimming, and (C) climbing over 15 min in the FST in SD and WKY rats (Experiment 2). Data are expressed as median with IQR, n = 10-11/group. Cyp: Cyprodime, FST: forced swim test, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

2.3.3. Experiment 3

2.3.3.1. Effects of morphine and cyprodime, alone or in combination, on general exploratory behaviour and defaecation during the pre-formalin trial in WKY and SD rats

Following drug injections, rats were habituated in the formalin arena for 10 min (pre-formalin trial) prior to receiving formalin injection and general exploratory behaviours and defaecation were recorded during this period (Table 2.6). Two-way ANOVA revealed significant effects of strain ($F_{1,103}$=60.964, ***p*<0.001**) and treatment ($F_{5,103}$=4.184, ***p*<0.01**), but not of strain x treatment interaction ($F_{5,103}$=0.556, p>0.05), on distance moved but further *post hoc* SNK test indicated there were no significant differences between relevant groups. KW test showed a significant main effect ($\chi^2(11)$=54.071, ***p*<0.001**) on rearing. *Post hoc* analysis revealed that vehicle-treated WKY rats exhibited significant reduction in duration of rearing, compared to SD counterparts (WKY-Vehicle vs SD-Vehicle, ***p*<0.001**). Systemic administration of the drugs, morphine and cyprodime, alone or in combination, did not affect rearing in either strain at the doses tested. KW analysis did not reveal any significant effects on grooming ($\chi^2(11)$= 18.728, p>0.05) or faecal pellets produced ($\chi^2(11)$=14.312, p>0.05) during pre-formalin trial.

Table 2.6: Effects of morphine (0.5 or 2 mg/kg, s.c.) and cyprodime (0.3 mg/kg, s.c.), alone or in combination, on general exploratory behaviours (distance moved, rearing, and grooming) and defaecation (number of pellets produced) during the 10 min period prior to formalin injection (pre-formalin trial). Data are expressed as mean ± SEM except for defaecation which is expressed as median with IQR, n=8-10/group. $^{###}p<0.001$ (WKY vs SD counterpart). Cyp: Cyprodime, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Groups	Distance moved (cm)	Rearing (s)	Grooming (s)	Defaecation
SD-Vehicle	1793.02±91.08	105.11±12.86	9.28±4.40	0 (0)
SD-Morph 0.5 mg/kg	1785.33±102.91	78.79±7.71	25.20±10.20	0 (0)
SD-Morph 2 mg/kg	1346.26±199.67	39.72±14.09	6.33±4.09	0 (0)
SD-Morph 0.5 mg/kg + Cyp 0.3 mg/kg	1899.07±191.00	93.32±13.12	8.74±3.27	0 (0)
SD-Morph 2 mg/kg + Cyp 0.3 mg/kg	2065.97±175.61	93.52±12.59	8.78±2.75	0 (0)
SD-Cyp 0.3 mg/kg	1897.17±85.86	94.44±9.91	28.90±9.20	0 (0)
WKY-Vehicle	1259.09±104.18	**33.54±5.81**$^{###}$	5.01±2.74	0 (1)
WKY-Morph 0.5 mg/kg	1258.52±83.08	32.21±9.58	12.37±3.47	0 (0)
WKY-Morph 2 mg/kg	942.67±111.24	40.74±9.51	8.15±3.00	0 (0)
WKY-Morph 0.5 mg/kg + Cyp 0.3 mg/kg	1126.60±82.70	38.44±9.07	9.33±8.57	0 (0)
WKY-Morph 2 mg/kg + Cyp 0.3 mg/kg	1230.05±83.17	30.57±5.81	13.20±4.47	0 (0)
WKY-Cyp 0.3 mg/kg	1238.55±123.24	32.25±5.35	3.49±2.78	0 (1)

2.3.3.2. Effects of morphine and cyprodime, alone or in combination, on formalin-evoked nociceptive and general exploratory behaviours in WKY and SD rats

Following the pre-formalin trial, rats received an intraplantar injection of formalin into the right hindpaw and were observed for 60 min. The CPS data were analysed temporally in 5 min time bins (Figure 2.10). Two-way RM ANOVA revealed significant effects of time ($F_{6.139,583.173}$=72.475, ***p*<0.001**), time x strain ($F_{6.139,583.173}$=7.350, ***p*<0.001**), and time x treatment ($F_{30.693,583.173}$=2.705, ***p*<0.001**) in tests of within-subjects effects and significant overall effects of strain ($F_{1,95}$=40.140, ***p*<0.001**) and treatment ($F_{5,95}$=8.092, ***p*<0.001**) in tests of between-subjects effects on formalin-evoked nociceptive behaviour. Further *post hoc* SNK analysis showed that vehicle-treated WKY rats exhibited higher formalin-evoked nociceptive behaviour in the first 15 min post-formalin injection, compared to SD counterparts ($T_{5\text{-}10}$: WKY-Vehicle vs SD-Vehicle, ***p*<0.05**; Figure 2.10A). Figures 2.10A and 2.10B show the effects of subcutaneous administration of morphine and/or cyprodime on nociceptive responding induced by formalin in SD and WKY rats, respectively. Morphine (0.5 and 2 mg/kg) significantly reduced formalin-evoked nociceptive behaviour in a dose-related manner in the SD ($T_{15\text{-}20}$: SD-Morphine 0.5 mg/kg vs SD-Vehicle, ***p*<0.05**; $T_{15\text{-}20}$, $T_{20\text{-}25}$, $T_{25\text{-}30}$, and $T_{30\text{-}35}$: SD-Morphine 2 mg/kg vs SD-Vehicle, ***p*<0.05**), but not in the WKY, rats. Morphine-induced antinociception was attenuated by pretreatment with cyprodime in SD rats ($T_{15\text{-}20}$ and $T_{20\text{-}25}$: SD-Morphine 2 mg/kg+Cyprodime 0.3 mg/kg vs SD-Morphine 2mg/kg, ***p*<0.05**). Cyprodime (0.3 mg/kg), administered alone, did not affect formalin-induced nociceptive behaviour in either strain. Formalin injection induced oedema in the right hindpaw in both strains (Figure 2.11) but two-way ANOVA revealed there was no significant effect of strain ($F_{1,106}$=3.412, p>0.05), treatment ($F_{5,106}$=2.102, p>0.05), or strain x treatment interaction ($F_{5,106}$=2.038, p>0.05) on paw oedema.

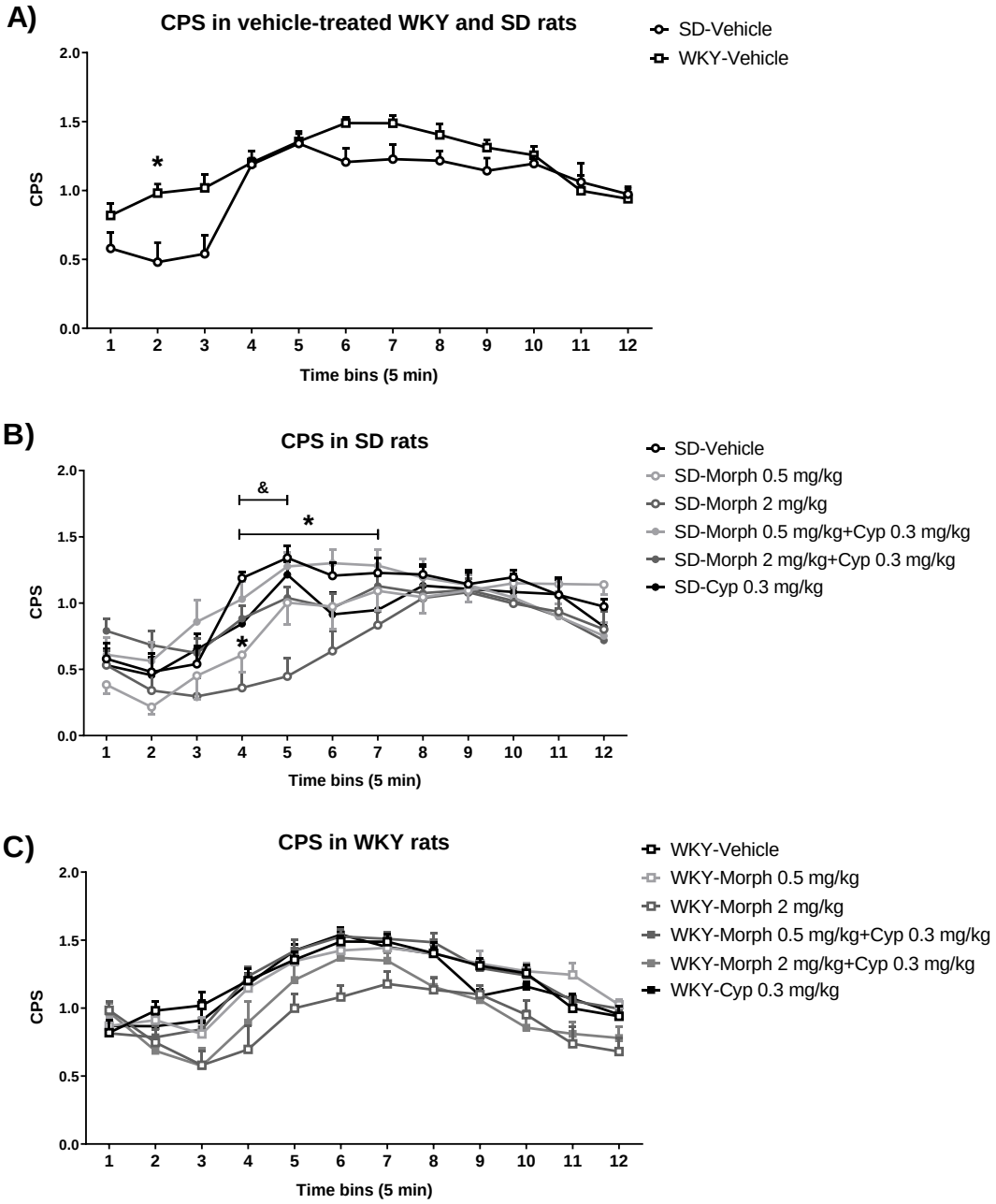


Figure 2.10: (A) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving vehicle injection systemically. Temporal profile of the effects of morphine (0.5 or 2 mg/kg, s.c.) and cyprodime (0.3 mg/kg, s.c.), alone or in combination, on formalin-evoked nociceptive behaviour in (B) SD and (C) WKY rats (Experiment 3). Data are presented in 5 min time bins and expressed as mean ± SEM, n = 8-10/group. *p<0.05 (SD-Morph 0.5 mg/kg or SD-Morph 2 mg/kg or WKY-Vehicle vs SD-Vehicle), $^{\&}p$<0.05 (SD-Morph 2 mg/kg+Cyp 0.3 mg/kg vs SD-Morph 2 mg/kg). CPS: composite pain score, Cyp: Cyprodime, Morph: morphine, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

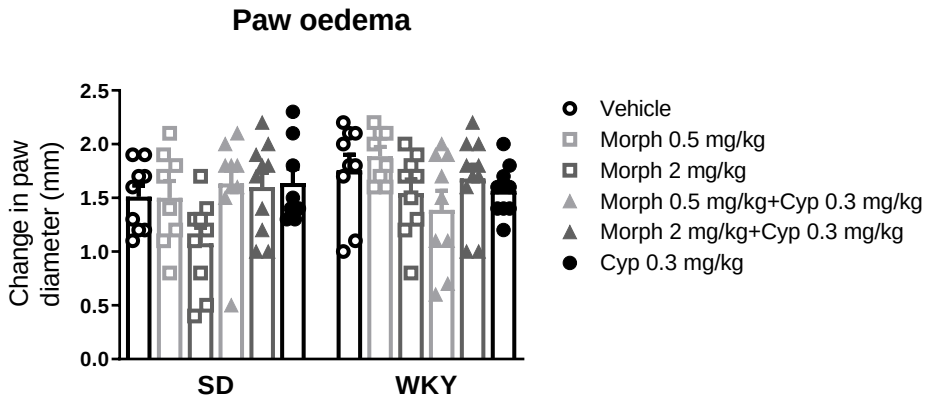


Figure 2.11: Effects of morphine (0.5 or 2 mg/kg, s.c.) and cyprodime (0.3 mg/kg, s.c.), alone or in combination, on formalin-induced hindpaw oedema in SD and WKY rats (Experiment 3). Paw oedema was measured as the difference in diameter (mm) of the right hindpaw immediately before, and 60 min after, intraplantar formalin injection. Data are expressed as mean ± SEM, n = 8-10/group. Cyp: Cyprodime, Morph: morphine, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

General exploratory behaviour and defaecation were also recorded and rated during the 60 min formalin trial (Table 2.7). A significant main effect ($\chi^2(11)=70.531$, $\boldsymbol{p<0.001}$) was observed on distance moved in the arena in KW test; *post hoc* test revealed that WKY rats, irrespective of drug/vehicle treatment, exhibited reduced distance moved during formalin trial compared to their SD counterparts. No significant main effect on rearing ($\chi^2(11)=19.291$, $p>0.05$), grooming ($\chi^2(11)=32.853$, $p>0.05$), or defaecation ($\chi^2(11)=36.401$, $p>0.05$) was revealed in KW analysis.

Table 2.7: Effects of morphine (0.5 or 2 mg/kg, s.c.) and cyprodime (0.3 mg/kg, s.c.), alone or in combination, on general exploratory behaviours (distance moved, rearing, and grooming) and defaecation (number of pellets produced) during the 60 min formalin trial. Data are expressed as mean ± SEM except for defaecation which is expressed as median with IQR, n=8-10/group. $^{\#\#}p<0.01$ (WKY vs SD counterpart). Cyp: Cyprodime, Morph: morphine, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Groups	Distance moved (cm)	Rearing (s)	Grooming (s)	Defaecation
SD-Vehicle	4763.23±358.02	5.70±2.27	16.96±4.24	4 (4)
SD-Morph 0.5 mg/kg	4045.76±404.22	0.85±0.48	20.35±8.88	0(0.75)
SD-Morph 2 mg/kg	3497.18±464.51	7.91±5.50	16.19±8.38	0(1)
SD-Morph 0.5 mg/kg + Cyp 0.3 mg/kg	3312.93±329.71	3.23±2.87	17.69±5.32	1(2.5)
SD-Morph 2 mg/kg + Cyp 0.3 mg/kg	3986.15±532.62	1.57±1.03	9.02±2.95	0(0.25)
SD-Cyp 0.3 mg/kg	3754.39±392.04	11.43±4.47	30.25±14.84	3(5)
WKY-Vehicle	**1714.68±80.54**$^{\#\#}$	3.34±2.96	30.87±3.49	0(0)
WKY-Morph 0.5 mg/kg	**1817.07±123.23**$^{\#\#}$	0.05±0.05	31.18±7.25	0(3)
WKY-Morph 2 mg/kg	**2003.84±248.29**$^{\#\#}$	4.43±1.94	56.96±8.37	0(0)
WKY-Morph 0.5 mg/kg + Cyp 0.3 mg/kg	**1711.39±119.10**$^{\#\#}$	0.84±0.64	36.40±6.99	0(0)
WKY-Morph 2 mg/kg + Cyp 0.3 mg/kg	**1976.30±160.03**$^{\#\#}$	1.98±1.03	50.54±11.94	0(0)
WKY-Cyp 0.3 mg/kg	**1819.06±119.72**$^{\#\#}$	1.16±0.72	39.76±11.44	0(0.5)

2.3.4. Levels of morphine in the plasma of WKY and SD rats

Levels of morphine were assayed in plasma from the WKY and SD rats (Table 2.8). KW test revealed a significant main effect ($\chi^2(11)=47.722$, ***p*<0.001**) on the plasma morphine level. Further *post hoc* tests revealed significant differences only between groups that received morphine systemically compared to vehicle counterparts in both SD (SD-Morphine 0.5 mg/kg or SD-Morphine 2 mg/kg vs SD-Vehicle, ***p*<0.001**) and WKY (WKY-Morphine 0.5 mg/kg or WKY-Morphine 2 mg/kg vs WKY-Vehicle, ***p*<0.001**) rats. However, no strain-related differences were observed in the plasma levels of circulating morphine.

Table 2.8: Level of circulating morphine in the plasma of SD and WKY rats. No difference was found in the plasma morphine levels between the two strains. Data = Mean ± SEM; n = 8-10/group. ****p*<0.001 (SD-Morphine 0.5 mg/kg or SD-Morphine 2 mg/kg vs SD-Vehicle), ^{+++}p<0.001 (WKY-Morphine 0.5 mg/kg or WKY-Morphine 2 mg/kg vs WKY-Vehicle). Morph: morphine, ND: not detected, s.c.: subcutaneous**,** SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Strain	Plasma concentration (ng/mL)		
	Vehicle, s.c.	Morphine 0.5 mg/kg, s.c.	Morphine 2 mg/kg, s.c.
SD	ND	18.64±2.34***	77.27±4.03***
WKY	ND	$18.88\pm1.20^{+++}$	$91.93\pm7.33^{+++}$

2.3.5. Expression of genes encoding MOP and POMC in brain regions associated with pain and negative affect in naïve WKY and SD rats

RT-qPCR was employed to determine if there was any strain difference in the basal levels in discrete brain regions (PFC, amygdala, hippocampus, hypothalamus, and PAG) for the expression of the genes encoding MOP and the prepropeptide POMC, the precursor protein for the endogenous MOP-preferential peptide β-endorphin. Analysis of gene expression revealed that WKY rats, compared to SD counterparts, exhibited higher levels of MOP ($t_{10.843}$=-2.298, ***p*<0.05**; Figure 2.12A) and POMC ($t_{10.791}$=-3.573, ***p*<0.01**; Figure

2.12B) mRNA in the hippocampus and PFC, respectively. No difference in the expression of the genes encoding MOP and POMC was observed in the other regions analysed between the two strains.

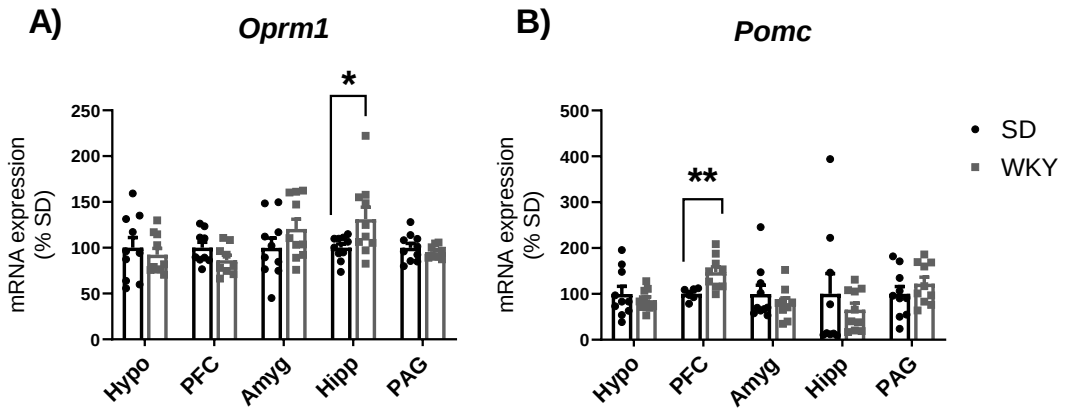


Figure 2.12: The expression of the genes encoding MOP and prepropeptide POMC in discrete brain regions involved in pain and negative affect in naïve WKY and SD rats. Data are expressed as mean ± SEM, n = 6-10/group. *p<0.05, **p<0.01 (WKY vs SD). Amyg: amygdala, Hypo: hypothalamus, Hipp: hippocampus, MOP: mu-opioid receptor, PAG: periaqueductal grey, PFC: prefrontal cortex, POMC: proopiomelanocortin, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

2.4. Discussion

In the current study we observed that systemic administration of morphine produced dose-dependent antinociception in the SD rats in tests of acute thermal (hot plate test) and persistent inflammatory (formalin test) pain, an effect attenuated by the selective MOP antagonist cyprodime. In comparison, WKY rats exhibited reduced responsivity to the antinociceptive effects of morphine in both pain assays employed here. Cyprodime did not affect nociceptive behaviour when administered alone in either strain. The study also provided some evidence for differential effects of morphine on anxiety- and depression-related behaviours in WKY versus SD rats. No difference was found in the circulating morphine level in the plasma of SD and WKY rats. Finally, RT-qPCR data showed region-dependent differential expression of the genes encoding MOP and POMC in the subcortical and limbic brain regions in behaviourally naïve WKY versus SD rats.

In the dose response experiment, dose-dependent antinociceptive effects of morphine treatment (0.5-7.5 mg/kg) were apparent in both SD and WKY rats in the hot plate test, although the minimum effective dose varied between the two strains. A higher dose of 2 mg/kg was required to produce significant antinociception in the WKY rats compared to the dose of 0.5 mg/kg in the SD counterparts. Consistent with our findings, previous studies have found WKY rats to be less sensitive to opioids (such as morphine and buprenorphine) than SD rats in acute thermal nociceptive assays (Sitsen et al., 1987; Hoffmann et al., 1998; Terner et al., 2003; Hestehave et al., 2019b). Of note, the WKY rats in our study did not display greater nociceptive responding on the hot plate at baseline, compared to SD counterparts, in contrast to a previous report (Burke et al., 2010). Methodological differences between the two studies and hypersensitivity exhibited by WKY rats to the stress of novel (aversive) environment exposure (Pardon et al., 2002) may account for the discrepancies observed here. It is also worth noting here that several other studies, including a recent one from Hestehave and colleagues, also did not find a difference in thermal response latency between WKY and SD rats (Hoffmann et al., 1998; Plesan et al., 1999; Taylor et al., 2001; Hestehave et al., 2019b).

The vehicle-treated WKY rats did exhibit an enhanced formalin-evoked nociceptive response particularly in the early phase of the test, compared to SD counterparts, thus confirming their hyperalgesic phenotype to noxious inflammatory stimulus as previously demonstrated by other studies (Burke et al., 2010; Rea et al., 2014). Though this finding is in contrast to one study showing no difference in formalin-evoked nociceptive

behaviour between WKY and SD rats (Taylor et al., 2001), the low concentration of formalin used by the authors (1.25% versus 2.5%) may partly explain the observed difference. Systemic administration of morphine resulted in significant dose-related antinociception in the SD, but not in the WKY, rats. The doses of morphine used in the formalin test component of the study (Experiment 3) were based on the results of the earlier dose response experiment (Experiment 1). We purposely chose low doses of morphine (0.5 and 2 mg/kg) for two reasons: 1) to avoid a "floor-effect" in both strains that might aid in unmasking any strain-related difference in morphine antinociception and 2) to avoid any potential effect of morphine on locomotor activity that might confound the results. Previous studies have reported significant antinociception produced by systemically injected morphine at low doses in the formalin test in SD (Munro, 2009; Boules et al., 2011) and Wistar (Biglarnia et al., 2013) rats, two of the most commonly used control strains for the inbred WKY rats. Our results indicate that WKY rats are hyporesponsive to morphine-induced antinociception in the formalin model of tonic, persistent inflammatory pain. Only one other study to date has compared morphine antinociception in the formalin assay between SD and WKY rats (Mahinda et al., 2004). However, the single dose of morphine used (3 mg/kg) was higher than our study, which might explain the lack of any differential effect of morphine on formalin-evoked nociceptive behaviour between WKY and SD rats in the study by Mahinda and colleagues. It appears that the reduced sensitivity to morphine in WKY versus SD rats may be due to a lack of adequate engagement of the MOP system to mediate antinociception. Consequently, it seems reasonable to suggest that such a deficit of the MOP system within descending inhibitory pain pathway may underpin hyperalgesia in the WKY rat strain. In addition, in the clinical context, our finding may suggest that MOP agonists could have reduced efficacy in patients presenting with comorbid pain and negative affect. However, the literature has conflicting results in this respect. Jamison et al has reported that the anxiety and depression scores of patients with low back pain is negatively correlated with efficacy of hydromorphone (an MOP agonist) and predictive of drug discontinuation (Jamison et al., 2013). In comparison, a recent study has demonstrated enhanced response to morphine is associated with patients with low back pain exhibiting greater depressive symptom and/or trait anxiety (Burns et al., 2017). Thus, further investigation is needed to fully understand the implication of our results in clinical setting in the context of comorbid pain and negative affect.

The WKY rats exhibited an overall reduced exploratory behaviour, compared to SD counterparts, during both pre- and post-formalin trials as measured by distance moved in the arena. In fact, this hypolocomotor behaviour is one of the hallmark traits of the WKY rat strain as reported previously in a number of studies (Paré, 1989c, 1994; Tejani-Butt et al., 1994; Burke et al., 2010) and has been linked to its behavioural inhibition phenotype (Paré, 1994; McAuley et al., 2009). No locomotor impairing effect of morphine was observed in pre- and post-formalin trials in either strain at the doses and times tested in our study. Previous studies showed that morphine reduced locomotor activity at doses ≥ 3 mg/kg in SD rats (Munro, 2009; Munro et al., 2010, 2012). In a recent study, mild reduction in locomotor activity by morphine 3 mg/kg was observed in both WKY and SD rats (Hestehave et al., 2019b). Thus, the behavioural differences in the effects of morphine in the formalin assay are likely to be related to a strain difference in nociceptive responsivity to the drug, rather than differential effects on locomotor activity. Another possibility behind such differential antinociceptive responses of morphine in WKY versus SD rats is that the pharmacokinetics of morphine are different in the two strains. However, the plasma morphine levels did not differ between SD and WKY rats, suggesting a strain-related pharmacokinetic difference is unlikely. Previous studies did not find differences in an array of pharmacokinetic parameters of systemic morphine in the plasma between spontaneously hypertensive rats and its normotensive control WKY rats (Bhargava and Villar, 1992), though brain levels of morphine were higher in the latter (Bhargava, 1982). Thus, we do acknowledge the limitation here that we did not determine all the pharmacokinetic parameters of morphine in the plasma or its level in relevant brain/spinal cord tissues between WKY and SD rats.

The present data also showed that cyprodime attenuated morphine-induced antinociception in the SD rats in both tests of nociception employed here. *In vitro* binding studies have shown that morphine preferentially binds to the MOP (Raynor et al., 1994; Mignat et al., 1995) although it exhibits affinity for both KOP and DOP (Chen et al., 1993; Meng et al., 1993; Toll et al., 1998). MOP is the major pharmacological target for morphine *in vivo* as shown by the complete absence of antinociceptive responses to morphine in MOP-null mice (Matthes et al., 1996). Cyprodime binds with high affinity (in the low nanomolar range) to the MOP and exhibits high selectivity for the MOP over KOP and DOP in both *in vitro* and *in vivo* assays (Schmidhammer et al., 1989; Márki et al., 1999). Moreover, presence of cyprodime reduced morphine-stimulated [^{35}S]GTPγS

binding by 500-fold in rat brain membranes (Márki et al., 1999). Thus, the attenuation of morphine-induced antinociception by cyprodime in SD rats further supports the contention that the behavioural responses of morphine observed in the present study were mediated via the MOP. In addition, the lack of any behavioural effect of systemically administered cyprodime alone in tests of both pain and negative affect in either strain suggests that the endogenous, basal MOP tone may not be different in SD and WKY rats.

The present study also investigated the effects of modulating MOP with morphine and cyprodime, alone or in combination, on anxiety-and depression-related behaviours in WKY and SD rats. It should be noted that exposure to tests (EPM/OF/FST) assessing behavioural responses in Experiment 2 did not yield any significant difference in the measures of anxiety- and depression-related behaviours in the control groups. A possible explanation for this finding could be habituation to the apparatus resulting in a loss of sensitivity of the key outcome measures (Nosek et al., 2008; Schrader et al., 2018). Henceforth, this discussion will focus mainly on the findings from the dose response experiment (Experiment 1). The vehicle-treated WKY rats did not exhibit significant difference in time spent or entries into open arms, compared to SD counterparts, which is in contrast with previously published reports (Gentsch et al., 1987; Paré, 1992; Malkesman and Weller, 2009) that demonstrated significant reduction in open arm activity in the WKY rat strain. Methodological differences in the design of the experiments (e.g. battery of behavioural tests) in our study and those published might account for this discrepancy. For instance, the hot plate tests which preceded the anxiety tests might affect the behaviours in the EPM in the WKY rats given that they are a stress-sensitive strain (Tejani-Butt et al., 1994; Zafar et al., 1997; Rittenhouse et al., 2002; De La Garza and Mahoney, 2004; Hyland et al., 2015). Acute stress has been shown to enhance freezing in the WKY rats (Paré, 1989c; Zafar et al., 1997; Nosek et al., 2008), which might affect the overall behaviour of these rats in the maze as observed here. In fact, this is reflected in the significant reduction in closed arm entries, a measure of spontaneous motor activity in the EPM test (Walf and Frye, 2007), in the vehicle-treated WKY versus SD rats. In the second test for anxiety-related behaviour that we used herein, the OF test, vehicle-treated WKY rats, in comparison to SD counterparts, displayed a significant reduction in entries made into the centre zone but no significant difference was observed in time spent in the centre zone of the arena. Previous reports from our laboratory (Burke et al., 2010, 2016) and others (Paré, 1994; Tejani-Butt et al., 1994; Pare

and Tejani-Butt, 1996; Will et al., 2003) have shown that the WKY rats spent significantly less time in the centre zone compared to SD rats. However, WKY rats, when exposed to a novel aversive (e.g. brightly lit) context, also exhibit increased immobility and higher latency to leave the centre of the OF compared to SD and other control rat strains (Paré, 1989c; Berton et al., 1997; O'Malley et al., 2010). We did observe that the vehicle-treated WKY rats took significantly longer to leave the centre compared to SD counterparts, which might partly explain the discrepancy here. Moreover, in accordance with previous reports (Paré, 1989b; Drolet et al., 2002; Pardon et al., 2002; Burke et al., 2010, 2016), vehicle-treated WKY rats exhibited pronounced hypolocomotor activity in the OF compared to SD counterparts, confirming their hypervigilant state and vulnerability to anxiety-related behaviours (Paré, 1994; McAuley et al., 2009). Vehicle-treated WKY rats also defaecated more in the OF arena compared to SD controls, further demonstrating their enhanced anxiety-like behaviour when exposed to a novel environment (O'Malley et al., 2010; Buckley et al., 2014; Hyland et al., 2015; Burke et al., 2016).

Systemic administration of morphine at various doses did not yield any significant anxiolytic effects in either strain. Higher doses of morphine (>5mg/kg) produced motor impairing effects in both SD and WKY rats as evidenced by significant reduction in open or closed arm entries in the EPM test. Using animal models of unconditioned and conditioned stimuli, several pharmacological studies reported anxiolytic effect of systemically administered morphine, particularly at low dose (0.3-1 mg/kg), in SD (Glover and Davis, 2008) and Wistar (Motta and Brandao, 1993; Anseloni et al., 1999; Kõks et al., 1999; Urigüen et al., 2002; Zarrindast et al., 2005) rats. To date, no study using morphine has been conducted in the WKY rat strain in this respect. However, one caveat is that not all of the studies cited above assessed general locomotor activity when morphine was used at high dose (e.g. ≥5 mg/kg) (Urigüen et al., 2002; Shin et al., 2003; Zarrindast et al., 2005; Glover and Davis, 2008). Interestingly, our data show that morphine at high doses differentially affected locomotor activity in the two strains – reducing distance moved in SD rats whereas increasing it in WKY counterparts. Morphine-induced effects on locomotor activity have been suggested to be mediated through the dopaminergic system (Zarrindast and Zarghi, 1992; Cook and Beardsley, 2003). Thus, the significance of such an effect between SD and WKY rats needs further probing to elucidate the underlying neurobiological differences in the two strains.

In the FST (Experiment 1), vehicle-treated WKY rats exhibited higher duration of immobility compared to SD counterparts, indicating increased behavioural despair, i.e. a depressive phenotype, as previously reported (Rittenhouse et al., 2002; Tejani-Butt et al., 2003; Burke et al., 2010, 2016; Nam et al., 2014). The motor-impairing effects of high doses of morphine were evident, particularly at the beginning of the trial, in both strains in the measure of duration of immobility. Morphine increased swimming duration with a concomitant reduction in climbing duration in both SD and WKY rats, though the latter effect failed to reach statistical significance in SD rats. Although the effects of acute pharmacological activation of MOP by morphine are less documented due to the adverse effects associated with this compound, the majority of these studies suggests morphine exerts an antidepressant effect (Besson et al., 1996; Broom et al., 2002; Zomkowski et al., 2005; Berrocoso et al., 2013; Falcon et al., 2015; Rosa et al., 2017). Of these, only one study that assessed effects of morphine in the FST in SD rats reported findings (increased swimming and decreased climbing) similar to our data here (Broom et al., 2002). However, it should be noted that the authors used the conventional two-day FST (pretest and test swim sessions) as compared to a single exposure in our study.

A difference in the expression and/or functionality of the MOP system (receptor and/or endogenous ligand) in the CNS may account for the differences in the phenotype and perhaps the antinociceptive potency of morphine between WKY and SD rats. We explored this possibility and measured basal expression of the genes encoding MOP and POMC in discrete brain regions in behaviourally naïve WKY and SD rats. There is considerable overlap in the neural substrates associated with pain and affect. The PFC, amygdala, and hypothalamus are key brain regions involved in processing sensory and/or affective-emotional components of pain, the hippocampus is implicated in processing mood, and the PAG is an important region within descending pain pathway for modulation of pain. Our data did not show a global difference in the expression profile of MOP and POMC mRNA between SD and WKY rats. Rather, the differences found in the expression of these genes were region-dependent: increased levels of MOP and POMC mRNA in the hippocampus and PFC, respectively, in WKY versus SD rats. A recent study by Burke et al showed higher expression of MOP mRNA in the hippocampus but no difference was found in the expression of POMC mRNA in the PFC in WKY versus SD rats (Burke et al., 2019). Methodological differences (e.g. singly- versus pair-housed naïve rats) may account for the discrepancy between these two studies. Both studies do

agree on the point that the differential expression of opioid-related genes in WKY versus SD rats is receptor/prepropeptide-specific and region-dependent. However, there are a few limitations here: firstly, differences in the expression of the genes may not translate to the protein level; secondly, potential strain-related alteration in the receptor functionality needs to be considered and investigated; and lastly, the large POMC prepropeptide is cleaved to other nonopioid peptides besides β-endorphin and so changes in POMC mRNA expression may not correlate to the level of β-endorphin. Thus, the significance of such region-dependent differences in the expression of genes encoding MOP and POMC requires further investigation, particularly at the level of endogenous peptide and the expression/functionality of the receptor protein in specific brain regions, to underpin the hyperalgesia and reduced morphine potency in the WKY rat strain.

In conclusion, the findings in this study demonstrate that the WKY rat has a hyperalgesic profile and vulnerability to both anxiety- and depression related behaviours compared to SD rats. In line with previous reports, WKY rats exhibited reduced responsivity to systemically administered morphine in an acute thermal assay of nociception. In addition, we showed for the first time that WKY rats displayed hyporesponsivity to systemic morphine in the formalin model of tonic persistent inflammatory pain, compared to SD counterparts. These differences in morphine sensitivity between the two strains are unlikely to be driven by strain-related differences in the pharmacokinetics of the drug. Using antagonist blockade, we further showed that morphine-induced antinociception was mainly mediated via the MOP. Effects of morphine on anxiety- and depression-related behaviours in both strains were less straightforward owing to its effect on locomotor activity. Though there are some region-dependent differences in the basal expression of the genes encoding MOP and POMC, further investigations are necessary to elucidate possible molecular deficits and key neural substrates involved in the differential nociceptive effects mediated by the MOP in the WKY rat model.

3. Chapter 3: Effects of pharmacological modulation of mu-opioid receptors in the ventrolateral periaqueductal grey on formalin-evoked nociceptive behaviour in Wistar-Kyoto and Sprague-Dawley rats

3.1. Introduction

The mesencephalic periaqueductal grey (PAG) is a key brainstem region involved in the modulation of nociceptive responses but also plays a pivotal role in coordinating defensive and aversive responses to emotional (e.g. stress, fear, and anxiety) situations (Behbehani, 1995; Bandler and Keay, 1996; Benarroch, 2012b). Thus, the PAG is an important brain region in both nociception and associated psychological responses. The PAG presents a high degree of anatomical and functional organisation as demonstrated in cytoarchitectural (Beitz, 1985) and histochemical (Conti et al., 1988) studies. It consists of four well-defined longitudinal subcolumns in rats: dorsomedial, dorsolateral, lateral and ventrolateral divisions (Figure 3.1). Stimulation of the different subcolumns of the PAG results in distinct behavioural responses, allowing the organism to respond appropriately to potential threats and noxious stimuli (for review see Bandler and Shipley, 1994; Keay and Bandler, 2001).

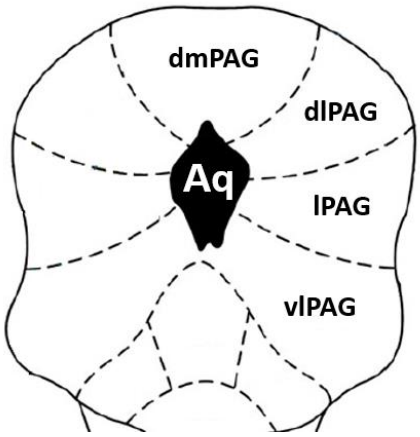


Figure 3.1: Divisions of PAG. Aq: Aqueduct, dmPAG: dorsomedial PAG, dlPAG: dorsolateral PAG; lPAG: lateral PAG; vlPAG: ventrolateral PAG. Figure adapted from Paxinos and Watson, 2007.

As previously mentioned in Chapter 1, the PAG is a major site for opioid-induced antinociception within the descending modulatory pain pathway (Reynolds, 1969; Mayer et al., 1971; Jacquet and Lajtha, 1974; Akil et al., 1976; Deakin and Dostrovsky, 1978; Llewelyn et al., 1983; Jensen and Yaksh, 1986; Bodnar et al., 1988; Smith et al., 1988; Fang et al., 1989a). Of its four functionally distinct subcolumns, the ventrolateral PAG (vlPAG) is important for mediating opioid-induced antinociception (Yaksh et al., 1976a; Cannon et al., 1982; Satoh et al., 1983; Jensen and Yaksh, 1986; Campion et al., 2016). The vlPAG contains a high density of MOP (Mansour et al., 1986, 1987; Tempel and Zukin, 1987; Commons et al., 1999, 2000; Wang and Wessendorf, 2002). Direct microinjection of morphine, the prototype drug for opioid analgesics, into the vlPAG produces robust antinociception in rats (Sharpe et al., 1974; Yaksh et al., 1976a; Lewis and Gebhart, 1977; Loyd et al., 2008; Bobeck et al., 2009; Morgan et al., 2009; Mehalick et al., 2013), which is reversed by systemic or site-specific administration of nonselective opioid antagonists, naloxone and naltrexone (Yaksh et al., 1976a; Hernandez-Leon et al., 2016). Moreover, pharmacological blockade of opioid receptors in the vlPAG attenuates the antinociceptive effects of systemically administered morphine (Lane et al., 2005). In addition, administering either MOP-selective antagonist β-FNA (Bobeck et al., 2012) or MOP-directed antisense oligodeoxynucleotide (Rossi et al., 1994a) into the same site prior to morphine blocked the antinociceptive effects of morphine injected into the vlPAG. In summary, these studies strongly indicate that the vlPAG is a key neural substrate mediating morphine-induced antinociceptive response via the MOP.

The stress-hypersensitive WKY rat strain is a genetic model of anxiety and depression (Paré and Redei, 1993; Tejani-Butt et al., 1994; Rittenhouse et al., 2002; Malkesman and Weller, 2009; Burke et al., 2010) that also displays heightened nociceptive response to noxious stimuli (Gunter et al., 2002; Zeng et al., 2008; Burke et al., 2010; O' Mahony et al., 2013; Rea et al., 2014), making it a useful tool for studying neurobiology of pain-negative affect interactions. Several studies implicate a dysregulation of the MOP system in the WKY phenotype (Avsaroglu et al., 2007; Browne et al., 2015; Burke et al., 2019). WKY rats exhibit reduced responsivity to morphine-induced antinociception in models of acute (heat) and chronic (inflammatory) pain, compared to SD counterparts (Hoffmann et al., 1998; Plesan et al., 1999; Terner et al., 2003; Hestehave et al., 2019b). In Chapter 2, we found that the WKY rats were hyporesponsive to the antinociceptive effects of systemically administered morphine in models of both phasic acute (hot plate test) and

tonic persistent inflammatory (formalin test) pain. However, no study to date has investigated the role of MOP within specific components of the descending pain pathway in the hyporesponsivity to MOP agonism exhibited by the WKY rats. Given the key role of the PAG in nociception and emotional coping strategies, it has been proposed as a possible neural substrate in mediating hyperalgesia associated with negative affect and stress (Madasu et al., 2016; Okine et al., 2017). Additionally, stress-induced hyperalgesia is associated with enhanced neuronal activity in the PAG in rats (Devall and Lovick, 2010; Sardi et al., 2018). Taken together, the ventrolateral subcolumn of the PAG is a region of interest with respect to investigating neurobiology of hyperalgesia co-occurring with negative affect in the WKY rat model.

The PAG modulates nociception via the descending pain pathway, relaying to the rostral ventromedial medulla (RVM), from which neurons project to the dorsal horn of spinal cord (DHSC) (Millan, 2002). Both behavioural and *in vitro* electrophysiology studies indicate that supraspinal MOP-induced antinociception is mediated in part by supressing the inhibitory influence of GABAergic interneurons within vlPAG onto the output neurons (Moreau and Fields, 1986; Depaulis et al., 1987; Chieng and Christie, 1994), leading to increased OFF cell and decreased ON cell activity in the RVM (Heinricher et al., 1992a, 1994). This GABAergic disinhibition of the descending PAG-RVM pathway inhibits nociceptive transmission at the level of DHSC (for review see Lau and Vaughan, 2014). Thus, examining neuronal activity at the level of RVM and DHSC following morphine injection into the vlPAG is useful in determining if any strain-related differences exist in the top-down modulation within the descending pain pathway. A valuable tool in visualising the pathways involved in the integration of a nociceptive input is mapping the expression of the nuclear protein c-Fos within neuronal nuclei (Harris, 1998; Coggeshall, 2005). Following a noxious stimulus, the immediate early gene *c-fos* is rapidly activated to express the protein in the populations of neurons excited by the nociceptive input (Hunt et al., 1987; Keay and Bandler, 1993; Abbadie et al., 1997). Hence, visualisation of activity within functionally-related neural substrates is possible due to the transient, rapid, and robust nature of the expression of c-Fos, establishing it as a marker of neuronal activity.

Therefore, this chapter examines the hypothesis that reduced MOP signalling in the vlPAG may underlie hyperalgesia to noxious inflammatory stimulus and

hyporesponsivity to MOP agonism in the WKY rats, compared to SD counterparts. Specific aims of the experiments described herein are as follows:

- To compare the effects of intra-vlPAG microinjection of the MOP agonist morphine on nociceptive responding to the tonic, persistent inflammatory noxious stimulus of intra-plantar-formalin injection in WKY versus SD rats.
- To examine any associated differences in expression of the marker of neuronal activity c-Fos in the RVM and DHSC (regions downstream of vlPAG).
- To determine if there are differences in the expression of MOP protein within vlPAG of WKY and SD rats.

3.2. Materials and methods

3.2.1. Animals

Male WKY and SD rats (8-10 weeks old, 220-250g on arrival; Envigo, Bicester, UK) were used in this study. Rats were maintained under a standard 12:12 h light/dark cycle (lights on from 07:00-19:00 h) in a temperature- (21±2°C) and humidity-controlled (45-55%) room throughout the study. Upon arrival, all animals were housed in groups of 3-4 per cage in plastic bottom cages (45x20x20 cm^3) containing 3Rs basic bedding (>99% recycled paper; 3Rs Lab, Fibrecycle Ltd, North Lincolnshire, UK) and sizzle nest material (LBS Biotechnology, Horley, UK) to acclimatise to the unit for 10 days. Animals were housed singly in cages with bedding and nesting material following surgery and remained singly-housed for the rest of the study. Food (14% protein rodent diet; Envigo, Bicester, UK) and water were available *ad libitum*. After surgery, a white rectangular plastic insert was placed into the cage under the food hopper allowing animals to access food freely but preventing them from getting too close to the cage top that might damage the implanted cannulae. A separate cohort of naïve WKY and SD rats were included in this study; they were singly-housed and did not undergo surgery or behavioural testing. All experimental procedures were carried out during the light phase between 08:00 and 18:00 h. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway. The work was carried out under license from the Health Products Regulatory Authority in the Republic of Ireland and in accordance with EU Directive 2010/63. The study was also designed and carried out in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

3.2.2. Chemicals and drug preparation

The MOP agonist morphine sulphate (60 mg/mL solution for injection; MercuryPharma, Surrey, UK) was purchased from a local pharmacy in Galway, Ireland. Morphine sulphate was dissolved in the vehicle 0.89% w/v NaCl (Fisher Scientific, Leicestershire, UK). As in Chapter 2, the salt form of morphine was taken into account and corrected for so that the final doses administered were equivalent to free base weight of morphine. On the test day, morphine sulphate solution was freshly prepared at concentrations 0.017, 0.043, and 0.086 μM and administered bilaterally into the vlPAG in an injection volume of 0.2 μL, giving the final doses of morphine administered as 1, 2.5, and 5 μg/0.2 μL/side. The doses of morphine were chosen based on previous studies demonstrating the antinociceptive

efficacy of the drug upon direct injection into the vlPAG in rats (Gogas et al., 1996; Zhu et al., 1997; Morgan et al., 2003; Lane et al., 2005; Hernandez-Leon et al., 2016; Tomim et al., 2016). Formalin (Sigma-Aldrich, Wicklow, Ireland) was also freshly prepared on the test day by diluting from a stock of 37% to 2.5% in sterile 0.89% NaCl.

3.2.3. Study design

The effects of pharmacological modulation of MOP in the vlPAG on formalin-evoked nociceptive behaviour in WKY and SD rats were investigated in this study. The experimental timeline is depicted in Figure 3.2. Animals underwent stereotaxic implantation of guide cannulae into the vlPAG under isoflurane anaesthesia. On the test day, animals were randomly assigned to treatment groups, resulting in 8 experimental groups as listed in Table 3.1. The sequence of treatments was also randomised to control for the order of testing and time of the day. Ten minutes after bilateral administration of vehicle (0.89% NaCl) or morphine (1, 2.5, or 5 μg) directly into the vlPAG, all rats received an intraplantar formalin injection into the right hindpaw under brief isoflurane anaesthesia. The rats were assessed in the formalin test for 90 min, after which they were euthanised by terminally anaesthetising with sodium pentobarbital (0.7 mL/kg, i.p.; Dolethal 200 mg/mL, Vetoquinol Ireland Ltd, Dublin, Ireland) and transcardially perfusing with heparinised saline followed by cold 4% paraformaldehyde (PFA). At the end of perfusion, brain and spinal cord were removed, post-fixed overnight in 4% PFA at 4°C, and then stored in cryoprotective solution (25% sucrose with 1% sodium azide in 0.1 M phosphate-buffered saline; Sigma-Aldrich, Wicklow, Ireland) until sectioning. The animals in the naïve cohort remained in their home cages until they were euthanised by decapitation. Whole brains were rapidly collected, snap frozen on dry ice and stored at -80°C for later use.

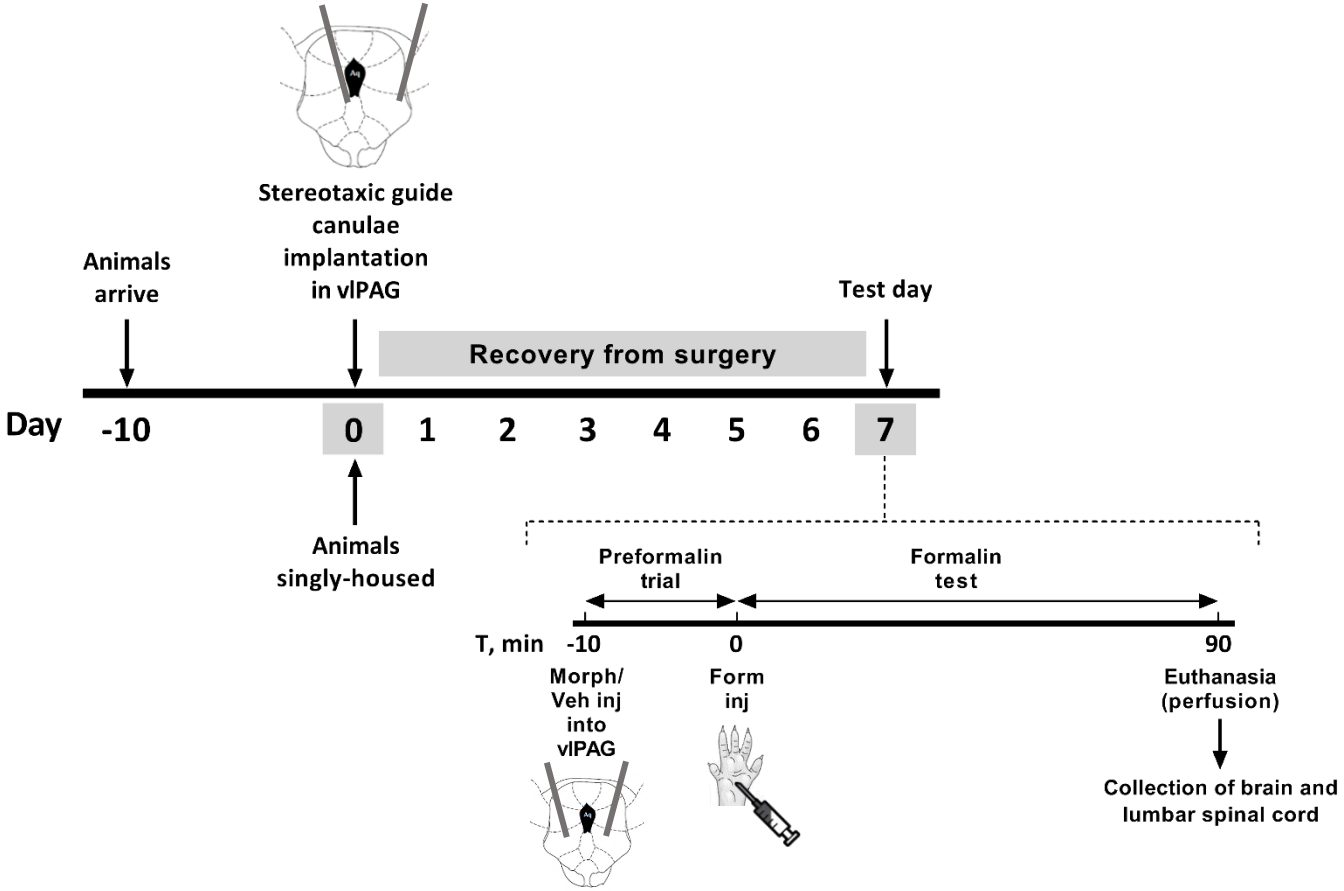


Figure 3.2: Schematic of the study outlining the design and timeline of behavioural test. Aq: Aqueduct, Form: formalin, Morph: morphine, T: time, Veh: vehicle, vlPAG: ventrolateral periaqueductal grey.

Table 3.1: Summary of experimental groups. n: number, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

Group	Strain	Treatment, intra-vlPAG	n	Final n after histological verification
1	SD	Vehicle	9	6
2	SD	Morphine 1 µg	9	6
3	SD	Morphine 2.5 µg	10	6
4	SD	Morphine 5 µg	9	6
5	WKY	Vehicle	10	7
6	WKY	Morphine 1 µg	10	6
7	WKY	Morphine 2.5 µg	9	6
8	WKY	Morphine 5 µg	9	7

3.2.4. Intra-vlPAG guide cannulae implantation

Following acclimatisation into the animal unit for 10 days, animals underwent stereotaxic implantation of stainless steel guide cannulae (length: 9 mm, 22G; Plastics One Inc, VA, USA) 1 mm above the vlPAG on both sides under isoflurane anaesthesia (3% induction and 2% maintenance in 0.5 L/min O_2). Rats were considered to be sufficiently anaesthetised when the tail-pinch withdrawal reflex was lost. The incisor bar was set at -3 mm for all surgeries. The coordinates for cannula implantation were as follows – SD: AP = ((difference from bregma to lambda) X 0.96 mm) from bregma, ML = ±1.9 mm at an angle of 10°, DV = 5.3 mm from the dura; WKY: AP = ((difference from bregma to lambda) X 0.974 mm) from bregma, ML = ±2.0 mm at an angle of 10°, DV = 5.4 mm from the dura, according to the rat brain atlas (Paxinos and Watson, 2007). The cannulae were permanently fixed to the skull using four stainless steel screws and carboxylate cement (Durelon^{TM}, MN, USA). A stainless steel stylet (length: 9 mm, 31G; Plastics One Inc, VA, USA) was inserted into each guide cannula to prevent blockage by debris. The nonsteroidal anti-inflammatory drug (NSAID), carprofen (2.5 mg/kg, s.c.; Rimadyl, Pfizer, Kent, UK), and the broad spectrum antibiotic, enrofloxacin (10 mg/kg, s.c.; Baytril, Bayer Ltd, Dublin, Ireland), were administered before surgery to manage postoperative pain and to prevent infection, respectively. Following implantation of cannulae, the rats were housed singly and enrofloxacin (10 mg/kg, s.c.) was administered once daily for a further 3 days. The rats were allowed to recover for at least 6 days post-

surgery before any behavioural testing. During this period, the rats were handled daily, stylets checked, and their body weights and general health monitored once daily.

3.2.5. Intra-vlPAG microinjection

On the test day, rats were gently restrained and received a bilateral microinjection (volume: 0.2 µL/side) of morphine (1, 2.5, or 5 µg) or vehicle (0.89% NaCl). A 28G injection needle was inserted through the cannula into the vlPAG that protruded 1 mm beyond the tip of the pre-implanted guide cannula. A 1 µL Hamilton microsyringe attached to a polyethylene tubing (length: ~50 cm, external diameter: 0.75 mm, internal diameter: 0.28 mm; Harvard Apparatus, Kent, UK) and a Harvard PHD2000 infusion pump (Harvard Apparatus, Kent, UK) was used for injection over a period of 60 s, as described previously (Finn et al., 2003; Rea et al., 2014). The needle was left in place for another 60 s before removal to allow diffusion of the drug and prevent any backflow through the guide cannula. Following microinjection, rats were placed directly in a darkened Perspex arena to habituate for 10 min (pre-formalin period).

3.2.6. Behavioural testing: Formalin-evoked nociceptive responding

Nociceptive responding to a persistent (tonic) noxious inflammatory stimulus was assessed in WKY and SD rats in the formalin test as described previously (Burke et al., 2010; Rea et al., 2014; Jennings et al., 2016; Okine et al., 2017). On the test day, following microinjection of vehicle or morphine into the vlPAG, rats were habituated in a darkened Perspex arena with clear base (30x30x40 cm^3, 30 lux) for 10 min prior to formalin injection (pre-formalin trial). To assess any effect of the drug on motor activity, the general exploratory (distance moved in the arena, rearing, and grooming) and aversive (jump) behaviours of the rats were recorded using a video camera located underneath the arena during this period. The rats then received an intraplantar formalin injection (2.5%, 50 µL) into the right hindpaw under brief isoflurane anaesthesia (3% induction in 0.8 L/min O_2) and were immediately returned to the same Perspex arena. The test began when the rat righted itself lasting for 90 min and nociceptive and motor-related behaviours were recorded. All behaviours were analysed later with the aid of EthoVision video tracking software (EthoVision XT, version 11.5; Noldus, Wageningen, Netherlands).

As described in Chapter 2 (section 2.2.4.1.2), formalin-evoked nociceptive behaviour was calculated using a weighted composite pain score (CPS) (Watson et al., 1997). Exploratory behaviours and number of faecal pellets produced during pre- and post-

formalin trials were also rated. The diameter of the formalin-injected hindpaw was measured using Vernier callipers immediately before injecting formalin and following euthanasia as a measure formalin-induced oedema. The arena was cleaned between animals with Milton cleaning solution.

3.2.7. Transcardial perfusion

Terminally anaesthetised rats that had lost toe- and tail-pinch reflex were placed in the supine position on a metal grated tray. An incision was made in the abdomen just below the ribcage using scissors. The sternum was lifted with a pair of clamps and the connective tissue and muscle underneath, including the diaphragm, were cut to expose the heart. A 25-gauge blunted needle, which was connected to an infusion pump delivering heparinised saline (5000 I.U. of heparin per litre of 0.89% NaCl; Wockhardt Ltd, Wrexham, UK) at a rate of 70 rpm (~24.5 ml/min), was inserted into the left ventricle of the heart and clamped in position. The right atrium was quickly nipped to allow efflux of blood. The pump pressure was increased to 180 rpm (~60 ml/min) and the rat was perfused with heparinised saline for 2 min until the blood was clear and the liver changed colour from deep red to blotchy brown. The reservoir supplying the pump was then switched to cold 4% PFA (Serosep Ltd, Limerick, Ireland) for another 2 min. The rat was perfused for a further 5 min at 120 rpm (~42 ml/min). Fixation was indicated by vigorous muscular tremors in the extremities of the animal during the first few minutes of perfusing with PFA.

3.2.8. Verification of injection site

Following transcardial perfusion, 0.2 µL of 1% fast green dye (dissolved in 0.89% NaCl; Sigma-Aldrich, Wicklow, Ireland) was slowly injected through each cannula to mark the site of vlPAG microinjection. Brains were then removed, post-fixed in 4% PFA overnight, and stored in cryoprotective solution (25% sucrose with 1% sodium azide in 0.1 M phosphate-buffered saline). Before sectioning, brains were snap-frozen on dry ice and coronal brain sections (thickness: 30 µm) were then sliced in a cryostat (Leica Biosystems, Wetzlar, Germany). Sections containing the PAG (from AP -7.30 to -8.72 mm relative to bregma) were collected with the aid of rat brain atlas (Paxinos and Watson, 2007), mounted on glass slides, and examined under a light microscope to obtain precise location of microinjection sites.

3.2.9. Tissue harvesting

3.2.9.1. Brain

After marking the microinjection sites, the animals were decapitated and brains were harvested as described in Chapter 2 (see section 2.2.5.2), post-fixed in 4% PFA overnight, and stored in cryoprotective solution. Brains were snap-frozen on dry ice before sectioning in the cryostat.

3.2.9.2. Spinal cord

The skin on the back of the carcass was cut down the midline with a pair of scissors and removed by pulling back to expose the dorsal portion of the carcass. The muscle along the spine was removed using a razor blade and toothed forceps. After exposure of the spine, a small rongeurs was used to make a cut in the lower lumber spine at the level of the pelvic crests. Using the rongeurs, the dorsal portion of the thoracic and lumber vertebra was dissected from the ventral portion proceeding in a caudal-to-cranial direction, revealing the dorsal aspect of the spinal cord. The spinal cord was lifted slowly and carefully from the spinal cavity with a small curved forceps that was also used to cut any nerve attachments. Once extracted, transverse cuts were made to isolate the lumbar segment (containing L4-L6) of the spinal cord. The lumbar spinal cord sections were post-fixed in 4% PFA overnight, stored in cryoprotective solution, and snap-frozen on dry ice before sectioning in the cryostat.

3.2.10. Immunohistochemistry (IHC) of c-Fos in the RVM and DHSC

After collecting PAG sections for verification of microinjection site, the remaining brain containing the RVM (from AP -9.30 to -11.30 mm relative to Bregma) and the lumbar regions (L4-L6) of the spinal cord were sectioned in the cryostat, according to the rat brain atlas (Paxinos and Watson, 2007). RVM and spinal cord sections (thickness: 30 µm) were collected in 12 and 6 series, respectively, and stored in 0.1 M phosphate buffer (PB) containing 0.1% sodium azide. Free floating immunohistochemical staining of c-Fos was then carried out.

Sections were washed once in 0.1 M phosphate-buffered saline (PBS; Sigma-Aldrich, Wicklow, Ireland) for 5 min and quenched to eliminate endogenous peroxidase activity by incubating for 5 min in 3% hydrogen peroxide (Sigma-Aldrich, Wicklow, Ireland) and 10% methanol (Fisher Scientific, Leicestershire, UK) in deionised water. A subsequent

three 5 min washes in PBS were done and the sections were incubated at room temperature for 1 h with 3% normal goat serum (Abcam, Cambridge, UK) and 0.2% Triton-X (Sigma-Aldrich, Wicklow, Ireland) in PBS to block non-specific binding. Primary antibody directed against full length protein corresponding to human c-Fos amino acid 1-380 (rabbit polyclonal to c-Fos, 1:2000 in 1% normal goat serum/0.2% Triton X in PBS; cat#ab190289, Abcam, Cambridge, UK) was then added to the sections and incubated overnight at room temperature under constant gentle agitation. Sections were again given 3 x 10 min washes and incubated for 3 h in biotinylated goat anti-rabbit antisera (1:200 in 1% normal goat serum in PBS; cat#ab97049, Abcam, Cambridge, UK). Next the sections were incubated in the avidin-biotin-peroxidase complex (1:200, Vectastain Elite ABC Kit; Vector Laboratories Ltd, Peterborough, UK) for 2 h. The sections were then immersed in 0.02% 3,3-diaminobenzidine tetrachloride (DAB, 4 mg in 1 ml PB; Sigma-Aldrich, Wicklow, Ireland) containing 0.01% hydrogen peroxide until a brown colour developed (60 and 20 s for RVM and spinal cord sections, respectively). After another 3 x 5 min washes in phosphate buffer (PB; Sigma-Aldrich, Wicklow, Ireland), sections were mounted on gelatin-coated microscope slides (Thermo Scientific, Braunschweig, Germany) and left to dry for at least 24 h before being dehydrated in ascending series of ethanol (Lennox, Dublin, Ireland): 50%, 70%, and twice in 100%, being immersed for 5 min in each solution. Sections were then cleared in xylene (Fisher Scientific, Leicestershire, UK) for 5 min and placed in xylene (clean) for a second time for 5 min. The slides were coverslipped using DPX mountant (Thermo Scientific, Cheshire, UK) and left to dry in the hood for a minimum of 3 days.

Images of the immunostained sections were taken with an Olympus C-5060 Wide Zoom digital camera (Mason Technologies, Dublin, Ireland) linked to an Olympus X5 microscope (Mason Technologies, Dublin, Ireland). c-Fos positive immunoreactive cells were counted in the nucleus raphe magnus (NRM) in the RVM. Relevant levels of the spinal cord were identified with the aid of rat brain atlas (Paxinos and Watson, 2007); c-Fos positive immunoreactive neurons were counted in the superficial (I-II) and the non-superficial (III-VI) laminae of the dorsal horn of lumber spinal cord (L4-L6). The number of c-Fos positive cells for each rat was calculated as the mean of three representative sections and an overall mean was then calculated for each treatment group. Counting was done manually in the regions of interest using the "cell counter" tool of NIH Image J1.52p software (Bethesda, MD, USA) and data were exported to Excel for final analysis.

3.2.11. Western immunoblot analysis of the expression of MOP in vlPAG

vlPAG tissue was obtained from fresh-frozen brains of naïve SD and WKY rats using the punch-microdissection method as described previously (Rea et al., 2014). Frozen coronal brain sections containing the vlPAG (from AP -7.64 to -8.72 mm relative to bregma) were sliced on the cryostat. A series of sections (thickness: 300 µm) were punched using cylindrical brain punchers (internal diameter: 0.75 mm; Harvard Apparatus, MA, USA). The samples were weighed (3±0.3 mg) and stored at -80°C before extracting protein for Western immunoblotting.

vlPAG tissue was lysed by brief sonication in 75 µL radioimmunoprecipitation (RIPA) lysis buffer [150 mM NaCl, 25 mM Tris-HCl (pH 7.6), 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM Na_3VO_4, 10 mM NaF containing 1% protease inhibitor cocktail (Sigma-Aldrich, Wicklow, Ireland) at a ratio of 1:10 (w/v)] in a 1.5 mL microcentrifuge tube. Nucleoprotein complexes were completely dissociated by placing the tissue homogenate on a shaker for 45 min at 4°C with constant agitation. The homogenate was then centrifuged at 14000*g* for 20 min at 4°C (Eppendorf Centrifuge 5415R, Stevenage, UK). The supernatant was collected and total protein content determined using Bradford assay. Bovine serum albumin (BSA) protein (Sigma-Aldrich, Wicklow, Ireland) standards (0, 0.0125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 mg/ml) were prepared in deionised water. In the Bradford assay, 250 µL Bradford reagent was added to 5 µL standards or unknown protein samples in triplicate on a 96-well optical plate. After a 10 min incubation period, absorption at 570 nm wavelength was determined for each sample. Protein concentrations of the samples were then determined using an 8-point standard curve constructed using the BSA standards. The samples were then equalised to 1.5 mg/mL protein concentration.

A 36 µg protein sample in sample loading buffer [4X sample loading buffer containing 25% v/v 1 M Tris HCl (pH 6.8), 5% w/v SDS, 20% v/v glycerol, 2.5% Bromophenol blue (0.2% w/v in 100% ethanol), and 20% v/v of 2-mercaptoethanol, made up to a total volume of 20 mL in deionised water] was boiled at 95°C for 5 min, briefly centrifuged, and subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 120 V for 2 h. The separated protein samples were electroblotted onto a nitrocellulose membrane (GE Healthcare Life Sciences, Freiburg, Germany) at 100 V for 50 min. Ponceau staining (0.1% ponceau dye in 5% acetic acid; Sigma-Aldrich, Wicklow, Ireland) was used to assess the efficiency of protein transfer. The membrane was washed

with deionised water to remove the stain and blocked in 5% non-fat powder milk (Aptamil®, Nutricia, Dublin, Ireland) in 0.1% Tris-buffered saline/Tween20 (TBST) solution for 1 h at room temperature to prevent non-specific binding. Primary antibody targeting the C-terminal amino acids 384-389 of rat MOP (rabbit polyclonal to MOP, 1:1000 in 5% milk/0.1% TBST; cat#ab10275, Abcam, Cambridge, UK) was added to the membrane and incubated for 1 h at room temperature and then overnight at 4°C under constant gentle agitation. The membrane was then given 4 x 5 min washes in TBST and incubated with secondary antibody containing IRDye (800CW) conjugated goat anti-rabbit antisera (1:10000 in 1% milk/0.1% TBST; cat#926-32211, LI-COR Inc, Lincoln, NE, USA) for 2 h at room temperature in the absence of light. Another 4 x 5 min washes in TBST were performed before scanning the blot in a LI-COR imager (Lincoln, NE, USA) with an exposure time of 2 min for the targeted channel. The membrane was then stripped for 5 min in stripping buffer (25 mM glycine and 1% SDS in deionised water, pH 2), followed by 4 x 5 min washes in TBST, and blocked again for 1 h at room temperature with blocking solution (5% milk/ 0.1% TBST). The membrane was reprobed with mouse monoclonal antibody to β-actin (1:10000 in 5% milk/0.1% TBST; cat#A5441, Sigma-Aldrich, Wicklow, Ireland) for 1 h at room temperature and then overnight at 4°C. Four 5 min washes in TBST were performed after which the secondary antibody containing IRDye (680-LT) conjugated goat anti-mouse antisera (1:10000 in 1% milk/0.1% TBST; cat#926-68020, LI-COR Inc, Lincoln, NE, USA) was added to the membrane and incubated for another 2 h at room temperature in the absence of light. The membrane was washed with TBST as before and scanned in the LI-COR imager. The LI-COR Image Studio™ imaging software (version 5.2) was used to automatically generate immunoreactive band intensities for expressions of MOP (~50 kDa) and β-actin (~42 kDa) proteins for each sample using the background subtraction method. The intensity ratio of MOP to β-actin was calculated for each sample, which was then normalised to the intensity ratio of the control group (SD) on a given blot; an overall mean was then calculated for each group.

3.2.12. Statistical analysis

The SPSS statistical software (IBM SPSS Statistics, version 24 for Windows; SPSS Inc, Chicago, IL, USA) was used to analyse all data. In all datasets the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was

considered an outlier and excluded from subsequent analysis. The normality and homogeneity of variance of the datasets were checked using Shapiro-Wilk and Levene's tests, respectively. The timecourse of formalin test was analysed with repeated measures (RM) ANOVA with strain and drug treatment as between-subject factors and time as within-subject factor. For RM ANOVA, sphericity of the datasets was checked with Mauchly's Test for Sphericity; if the assumption of sphericity was violated, a Greenhouse-Geisser correction was used. Behavioural data (except for jump and defaecation) and IHC results in the RVM were analysed using two-way analysis of variance (ANOVA) with strain and drug treatment as factors. The IHC results in the spinal cord were analysed with three-way ANOVA with ipsi-/contralateral (with respect to formalin injection) sides as an additional factor. *Post hoc* Student-Newman-Keuls (SNK) test was carried out for pairwise group comparisons, where appropriate. Student's unpaired, two-tailed t-test was employed to analyse the expression results of MOP in the vlPAG. The level of significance was deemed $p<0.05$. If the data were not normally distributed and/or the variance was not homogeneous, three transformations were applied, in the order: square root, natural logarithm, and ranking of the data values to evaluate if parametric statistics can be used. In addition, if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular dataset being analysed, parametric statistical approach was still employed (Moore et al., 2009).

If any dataset was ordinal (e.g. jump and defaecation) or found to be not normally distributed and/or the variance was not homogeneous even after transformation, non-parametric statistical analysis was performed using Kruskal-Wallis (KW) one-way analysis of variance by rank. *Post hoc* Mann-Whitney U-test (*p*-value was adjusted using Bonferroni-Holm correction for multiple comparisons) was carried out, where appropriate. In all cases statistical analysis of a dataset was carried out for all the groups together. However, in some instances, the groups are graphed separately to present the results clearly in a reader-friendly format. Data are expressed as either mean ± standard error of the mean (S.E.M) or median with interquartile range (IQR) depending on the statistical approach undertaken, parametric or non-parametric, respectively.

3.3. Results

3.3.1. Histological verification of injection site in vlPAG

Histological verification under the light microscope confirmed that 65% and 68% of the injections were correctly placed within the borders of the left and right vlPAG in SD and WKY rats, respectively (Figure 3.3). The remaining 35% and 32% of injections in SD and WKY rats, respectively, had either one or both cannulae placed in the lPAG or outside the PAG in the mesencephalic trigeminal nucleus. Only those rats in which the injections were accurately positioned within both left and right vlPAG were included in subsequent analysis (see Table 3.1 for final n in each group).

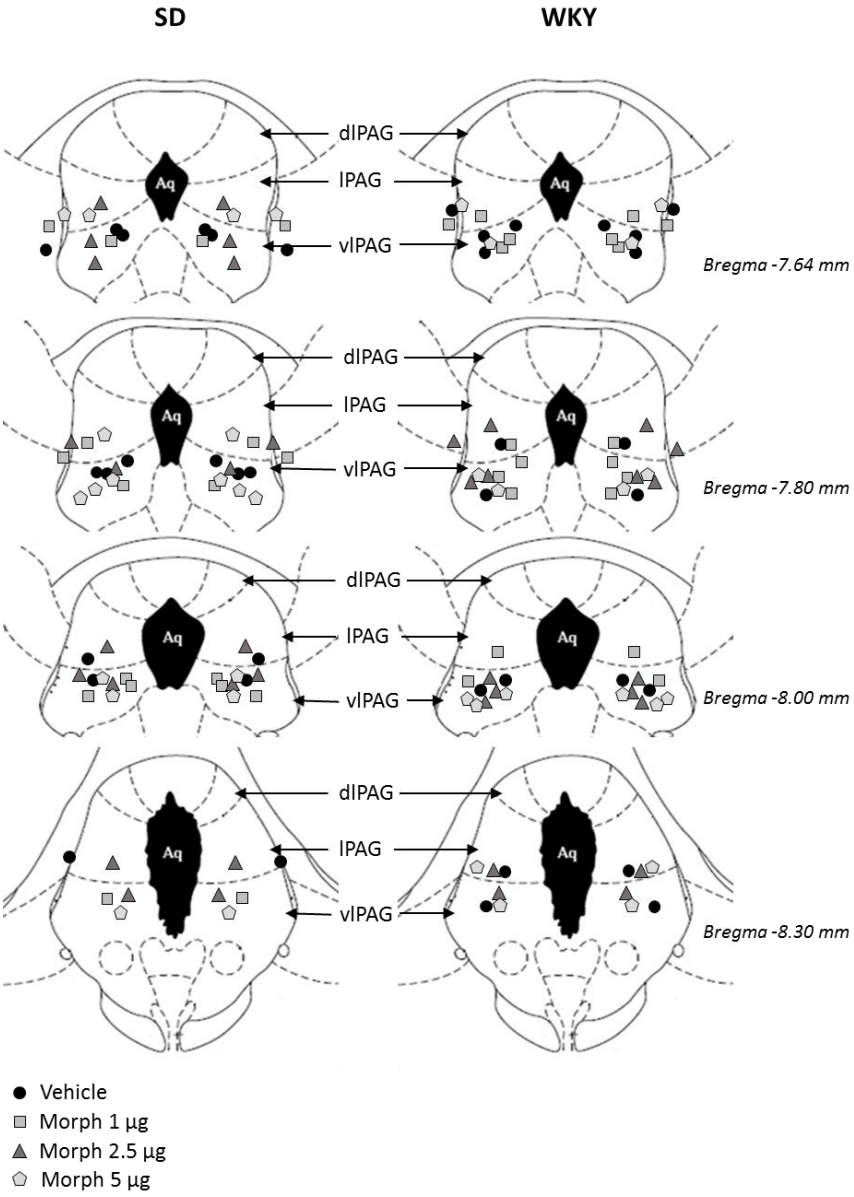


Figure 3.3: Schema of histological verification of microinjection sites of vehicle (●), morphine 1 µg (□), morphine 2.5 µg (△), and morphine 5 µg (⬠) in the vlPAG in SD (left panel) and WKY (right panel) rats (adapted from Paxinos and Watson, 2007). Aq: Aqueduct, dlPAG: dorsolateral periaqueductal grey, lPAG: lateral periaqueductal grey, Morph: morphine, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

3.3.2. Effects of bilateral intra-vlPAG administration of morphine on general exploratory behaviour and defaecation during pre-formalin trial in WKY and SD rats

Immediately after the intracerebral injection, rats were placed in the arena for 10 min and general exploratory behaviours including defaecation were recorded during this pre-formalin trial as described in Chapter 2 (Table 3.2). KW analysis revealed that there was no significant main effect ($\chi^2(7)=6.186$, $p>0.05$) on distance moved in the arena. A significant main effect ($\chi^2(7)=15.221$, $\mathbf{p<0.05}$) was observed in the KW test on rearing but further *post hoc* analysis indicated there were no significant differences between drug-treated groups within each strain or between the two strains. KW test did reveal a significant main effect ($\chi^2(7)=34.215$, $\mathbf{p<0.001}$) on grooming. *Post hoc* pairwise comparisons showed that morphine at higher doses (2.5 or 5 µg) significantly reduced grooming in SD (SD-Morphine 2.5 µg or SD-Morphine 5 µg vs SD-Vehicle, $\mathbf{p<0.01}$) and WKY (WKY-Morphine 5 µg vs WKY-Vehicle, $\mathbf{p<0.01}$) rats. KW analysis did not reveal any significant effect on number of jumps ($\chi^2(7)=13.062$, $p>0.05$). Though not statistically significant, it should be noted that a proportion of SD (morphine 2.5 µg: 1 out 6, morphine 5 µg: 2 out of 6) and WKY (morphine 2.5 µg: 1 out 6, morphine 5 µg: 2 out of 7) rats that received high doses of morphine exhibited jump response. Lastly, no significant main effect ($\chi^2(7)=7.209$, $p>0.05$) on faecal pellets excreted during pre-formalin trial was revealed in the KW test.

Table 3.2: Effects of bilateral intra-vlPAG administration of vehicle or morphine (1, 2.5, or 5 µg/200 nL/side) on general exploratory (distance moved, rearing, and grooming) and aversion-related (jump) behaviours and defaecation (number of pellets produced) during the 10 min pre-formalin trial immediately after bilateral intra-vlPAG microinjection. Data are expressed as mean ± SEM except for jump and defaecation which are expressed as median with interquartile range, n=6-7/group. ***p*<0.01 (SD-Morph 2.5 µg or SD-Morph 5 µg vs SD-Vehicle), ^{++}p<0.01 (WKY-Morph 5 µg vs WKY-Vehicle). Morph: morphine, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

Groups	Distance moved (cm)	Rearing (s)	Grooming (s)	Jump	Defaecation
SD-Vehicle	2080.86±631.71	42.38±9.56	35.50±8.12	0 (0)	0 (0)
SD-Morph 1 µg	1221.63±364.36	11.16±6.68	12.05±9.73	0 (0)	0 (0.5)
SD-Morph 2.5 µg	1327.01±1025.13	17.36±16.50	**0.00±0.00****	0 (8)	0 (0.25)
SD-Morph 5 µg	1777.61 ±720.02	20.09±7.75	**0.00±0.00****	0 (21)	0.5 (2.25)
WKY-Vehicle	1699.61±182.50	30.85±4.86	23.19±5.61	0 (0)	0 (0)
WKY-Morph 1 µg	1054.86±255.81	18.13±5.89	8.90±2.90	0 (0)	0 (0.25)
WKY-Morph 2.5 µg	1028.09±552.51	4.08±3.46	3.31±3.29	0 (8.25)	0 (0)
WKY-Morph 5 µg	1097.93±276.31	19.65±14.44	**0.00±0.00**$^{++}$	0 (27)	0 (1)

3.3.3. Effects of bilateral intra-vlPAG administration of morphine on formalin-evoked nociceptive and general exploratory behaviours in WKY and SD rats

Intraplantar formalin injection produced robust nociceptive behaviour (elevation, shaking, flinching, licking, and biting of the right hindpaw) in both WKY and SD rats as indicated by the CPS. The animals from both strains exhibited the classic biphasic response to intraplantar formalin injection: an early (first) phase characterised by acute nociceptive behaviour immediately after formalin injection lasting approximately for 10 min, and then after a brief quiescent period (interphase), a late (second) phase of nociceptive behaviour beginning 15 to 20 min after formalin injection (Barrot, 2012). CPA data were analysed temporally in 5 min time bins over the 90 min course of the trial (Figure 3.4). Two-way RM ANOVA revealed significant effects of time ($F_{3.807,159.902}$=3.062, ***p*<0.05**), time x strain ($F_{3.807,159.902}$=3.080, ***p*<0.05**), and time x treatment ($F_{11.422,159.902}$=2.075, ***p*<0.05**) in tests of within-subjects effects on formalin-evoked nociceptive behaviour. Additionally, significant overall effects of strain ($F_{1,42}$=36.538, ***p*<0.001**), treatment ($F_{3,42}$=56.263, ***p*<0.001**), and strain x treatment interaction ($F_{3,42}$=8.601, ***p*<0.001**) were obtained in tests of between-subjects effects on formalin-evoked nociceptive behaviour. Further SNK *post hoc* tests showed that WKY rats receiving intra-vlPAG vehicle microinjection exhibited higher formalin-evoked nociceptive behaviour for the first 30 min of the trial, compared to SD counterparts [T_{0-30} (in 5 min bins): WKY-Vehicle vs SD-Vehicle, ***p*<0.05**; Figure 3.4A]. Figure 3.4B and 3.4C illustrate the effects of bilateral intra-vlPAG administration of morphine at different doses on nociceptive responding induced by formalin in SD and WKY rats, respectively. Morphine 1 µg markedly attenuated formalin-evoked nociceptive behaviour in the SD rats [T_{15-60} (in 5 min bins): SD-Morphine 1 µg vs SD-Vehicle, ***p*<0.05**], whereas it had no such significant antinociceptive effect in the WKY counterparts. Morphine at higher doses (2.5 and 5 µg) were antinociceptive in both SD [T_{0-5}, T_{20-65} (in 5 min bins), and T_{70-75}: SD-Morphine 2.5 µg or SD-Morphine 5 µg vs SD-Vehicle, ***p*<0.05**) and WKY [T_{0-90} (in 5 min bins): WKY-Morphine 2.5 µg or WKY-Morphine 5 µg vs WKY-Vehicle, ***p*<0.05**] rats. Formalin injection induced oedema in the right hindpaw in both strains (Figure 3.5). Two-way ANOVA revealed no significant effect of strain ($F_{1,49}$=2.392, p>0.05), treatment ($F_{3,49}$=2.332, p>0.05), or strain x treatment interaction ($F_{3,49}$=0.838, p>0.05) on paw oedema.

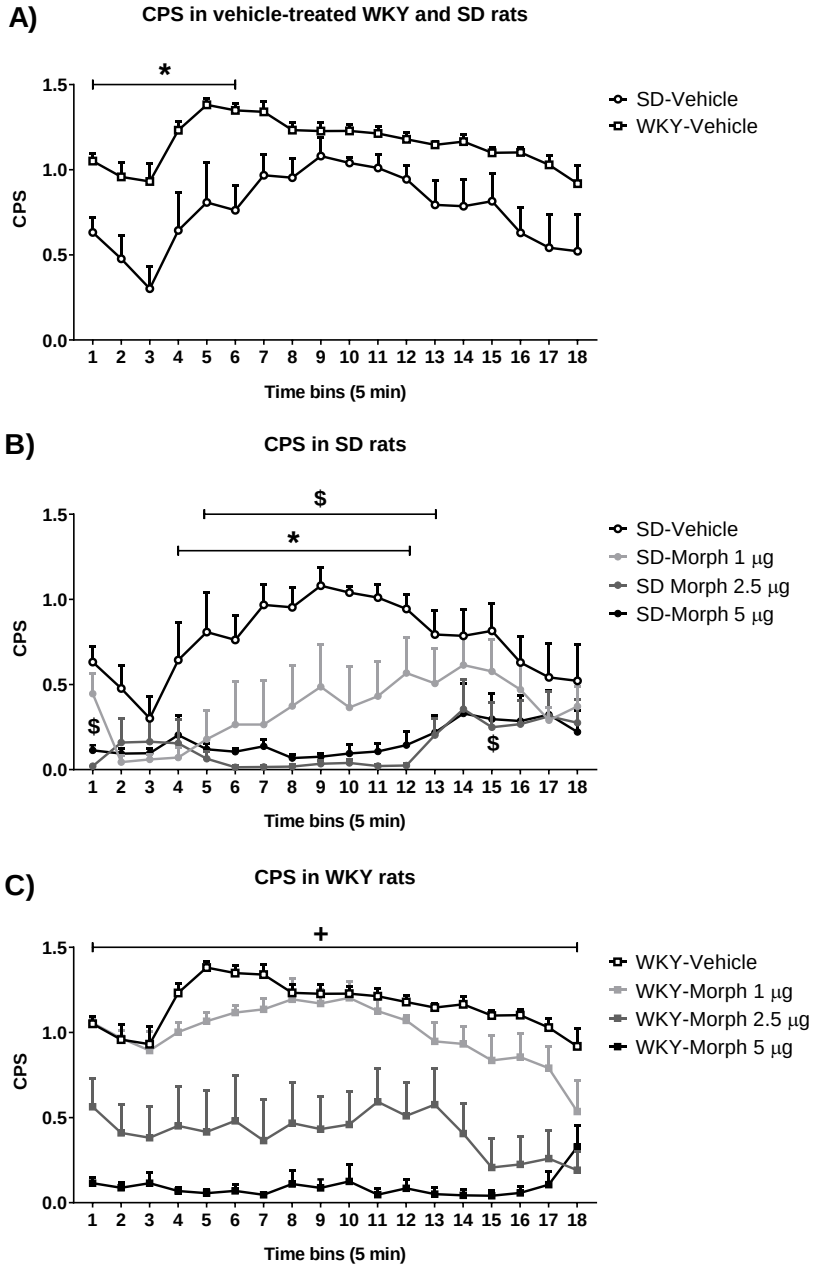


Figure 3.4: (A) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving intra-vlPAG microinjection of vehicle. Temporal profile of the effects of bilateral intra-vlPAG microinjection of morphine (1, 2.5, or 5 µg/200 nL/side) on formalin-evoked nociceptive behaviour in (B) SD and (C) WKY rats. Data are presented in 5 min time bins and expressed as mean ± SEM, n = 6-7/group. *p<0.05 (SD-Morph 1 µg or WKY-Vehicle vs SD-Vehicle), $^{\$}p$<0.05 (SD-Morph 2.5 µg or SD-Morph 5 µg vs SD-Vehicle), ^{+}p<0.05 (WKY-Morph 2.5 µg or WKY-Morph 5 µg vs WKY-Vehicle). CPS: composite pain score, Morph: morphine, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

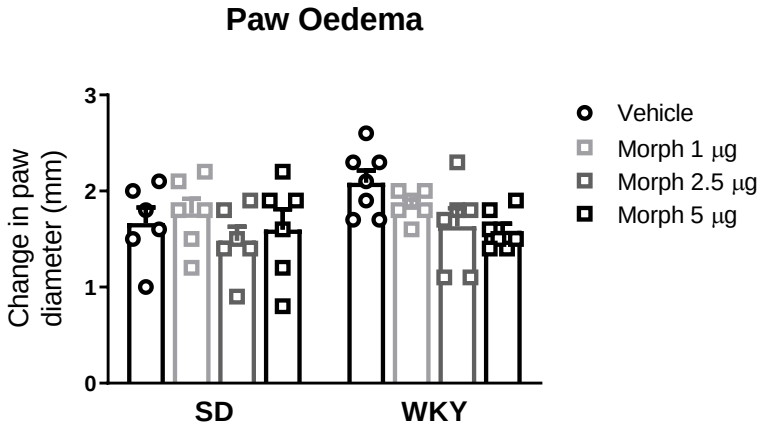


Figure 3.5: Effects of bilateral intra-vlPAG administration of vehicle or morphine (1, 2.5, or 5 µg/200 nL/side) on formalin-induced paw oedema in SD and WKY rats. Paw oedema was measured as the difference in diameter (mm) of the right hindpaw immediately before, and 90 min after, intraplantar formalin injection. Data are expressed as mean ± SEM, n = 6-7/group. Morph: morphine, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

General locomotor activity and defaecation were also analysed during the 90 min formalin trial (Table 3.3). KW test found a significant main effect ($\chi^2(7)=18.591$, ***p*<0.01**) on distance moved in the arena; however, further *post hoc* test indicated there were no significant differences between relevant groups. No significant main effect ($\chi^2(7)=7.591$, $p>0.05$) on rearing was found in KW analysis. KW test did reveal a significant main effect ($\chi^2(7)=32.344$, ***p*<0.001**) on grooming. *Post hoc* analysis showed that all three doses of morphine significantly reduced grooming in SD (SD-Morphine 1 µg or SD-Morphine 2.5 µg or SD-Morphine 5 µg vs SD-Vehicle, ***p*<0.01**) and WKY (WKY-Morphine 1 µg or WKY-Morphine 2.5 µg or WKY-Morphine 5 µg vs WKY-Vehicle, ***p*<0.01**) rats, compared to respective vehicle counterparts. With regards to aversion-related behaviour, KW test showed there was no significant main effect on number of jumps ($\chi^2(7)=8.289$, $p>0.05$) during formalin trial. Similar to pre-formalin trail, a proportion of SD (morphine 2.5 µg: 1 out 6, morphine 5 µg: 2 out of 6) and WKY (morphine 2.5 µg: 1 out 6, morphine 5 µg: 1 out of 7) rats receiving high doses of morphine exhibited jump response, although these results are not statistically significant. KW test showed a significant main effect

($\chi^2(7)=14.635$, ***p*<0.05**) on faecal pellets excreted during formalin trial but no significant differences between relevant groups were found in the *post hoc* test.

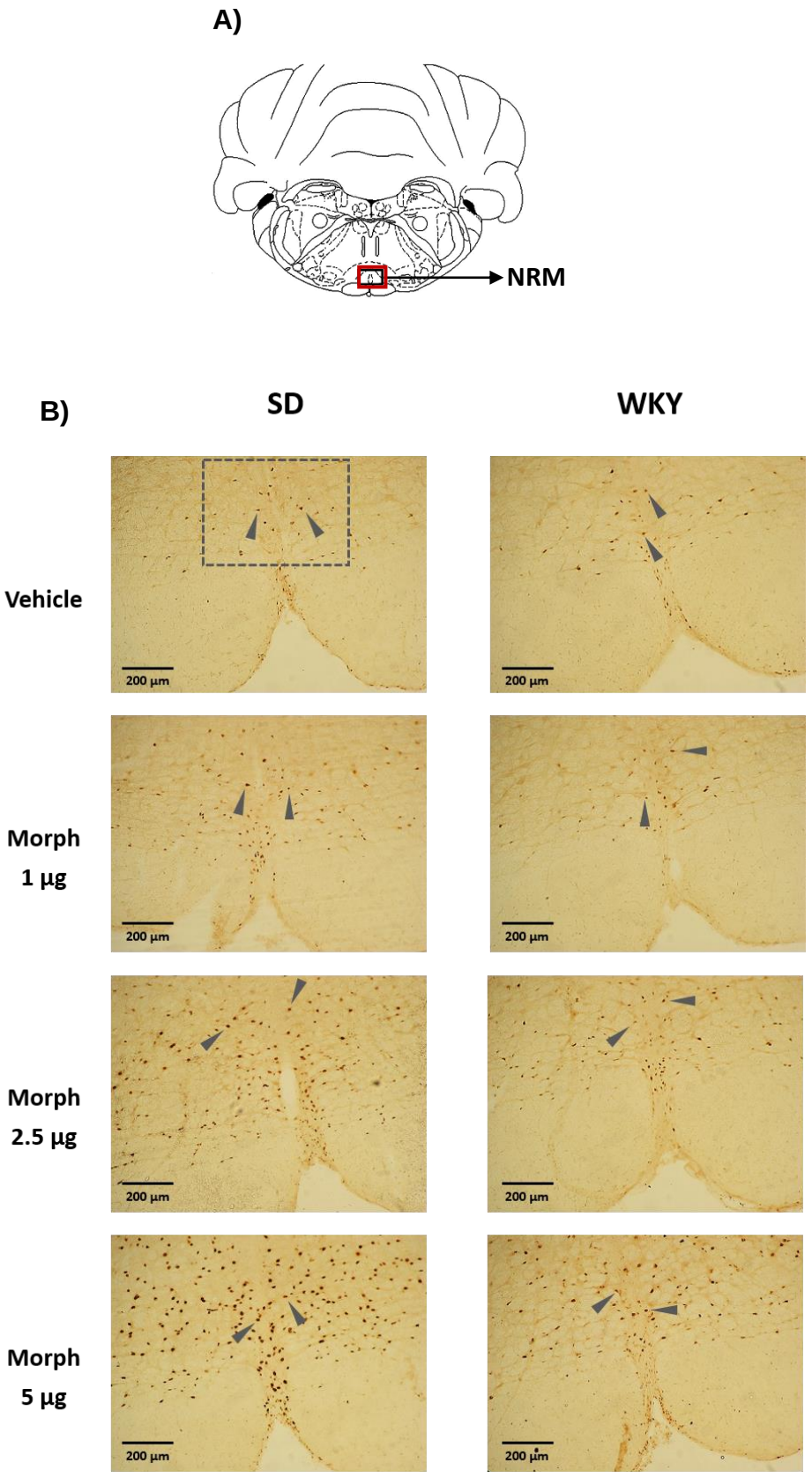
Table 3.3: Effects of bilateral intra-vlPAG administration of vehicle or morphine (1, 2.5, or 5 µg/200 nL/side) on general exploratory (distance moved, rearing, and grooming) and aversion-related (jump) behaviours and defaecation (number of pellets produced) during the 90 min formalin trial. Data are expressed as mean ± SEM except for jump and defaecation which are expressed as median with interquartile range, n=6-7/group. ***p*<0.01 (SD-Morphine 1 µg or SD-Morphine 2.5 µg or SD-Morphine 5 µg vs SD-Vehicle), ^{++}p<0.01 (WKY-Morphine 1 µg or WKY-Morphine 2.5 µg or WKY-Morphine 5 µg vs WKY-Vehicle). Morph: morphine, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

Groups	Distance moved (cm)	Rearing (s)	Grooming (s)	Jump	Defaecation
SD-Vehicle	8559.22± 3061.48	30.29± 13.99	128.20± 44.48	0 (0)	2 (4)
SD-Morph 1 µg	2599.85± 966.19	146.42± 105.45	**5.37± 3.51****	0 (0)	2 (6.25)
SD-Morph 2.5 µg	2789.69± 1580.65	36.51± 34.43	**5.28± 5.24****	0 (0.25)	0.5 (3.5)
SD-Morph 5 µg	5022.39± 569.18	15.14± 9.73	**0.51± 0.51****	0 (23)	1 (4)
WKY-Vehicle	2908.08± 407.05	0.81± 0.64	56.33± 17.23	0 (0)	0 (0)
WKY-Morph 1 µg	2496.65± 1369.87	7.49± 4.78	**5.41± 2.85**$^{++}$	0 (0)	0 (0.25)
WKY-Morph 2.5 µg	1865.20± 809.18	0.19± 0.19	**3.57± 2.86**$^{++}$	0 (3.75)	0 (0)
WKY-Morph 5 µg	1221.29± 296.04	6.72± 6.11	**0.03± 0.03**$^{++}$	0 (30)	0 (2)

3.3.4. Effects of bilateral intra-vlPAG administration of morphine on immunoreactive c-Fos-positive cells in RVM and dorsal lumbar spinal cord

3.3.4.1. RVM

The expression of c-Fos was measured in the nucleus raphe magnus (NRM) that is the rostral-most structure in the RVM and restricted to the midline, just above the pyramidal tract at the base of the brainstem (Figure 3.6A and 3.6B). Two-way ANOVA revealed a significant effect of treatment ($F_{3,47}$=32.798, ***p*<0.001**), but not of strain ($F_{1,47}$=0.278, p>0.05) or strain x treatment interaction ($F_{3,47}$=2.397, p>0.05), on the expression of c-Fos positive cells. Further *post hoc* SNK test showed that bilateral intra-vlPAG administration of morphine produced a dose-dependent increase in c-Fos expression in the NRM in SD and WKY rats, compared to respective vehicle counterparts (Figure 3.6C). Interestingly, the WKY rats exhibited a blunted increase in the NRM c-Fos expression induced by intra-vlPAG morphine, compared to SD rats (SD-Morphine 1 µg or SD-Morphine 2.5 µg or SD-Morphine 5 µg vs SD-Vehicle, ***p*<0.05**; WKY-Morphine 5 µg vs WKY-Vehicle, ***p*<0.05**).


A)
NRM
B)
SD
WKY
Vehicle
Morph
1 µg
Morph
2.5 µg
Morph
5 µg
200 µm

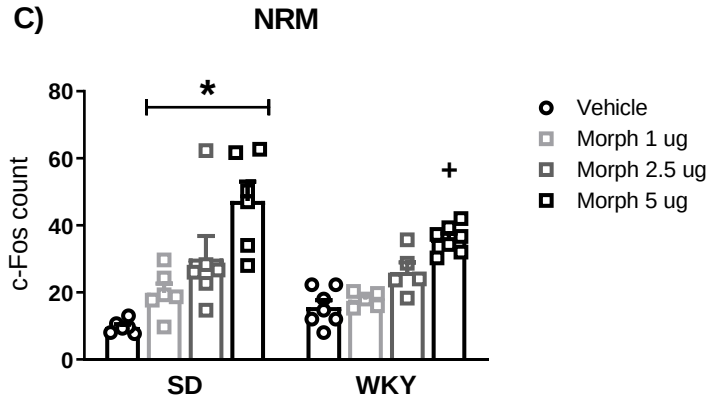
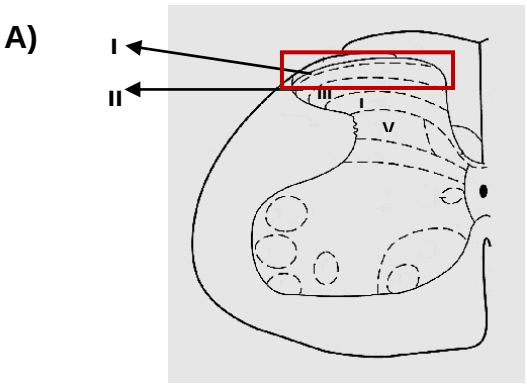


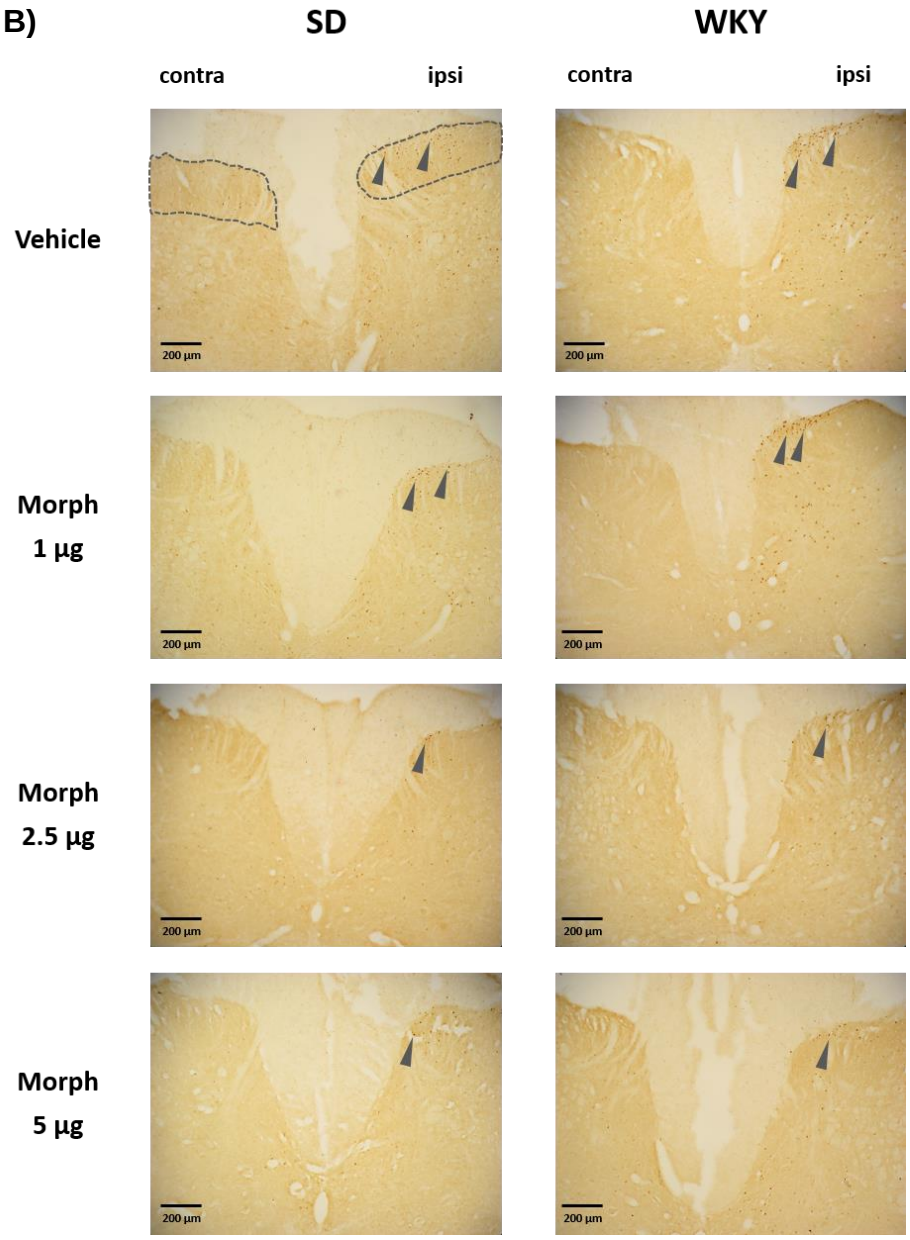
Figure 3.6: (A) Anatomical location of rat NRM (open square) in the RVM (adapted from Paxinos and Watson, 2007). (B) Representative images (10X magnification) of the expression of c-Fos positive cells in the NRM in SD (left panel) and WKY (right panel) rats receiving intra-vlPAG microinjection of vehicle or morphine. Grey arrows point out to some of the c-Fos positive cells in each section. Grey dotted box represents the area where c-Fos-like immunoreactive cells were counted. (C) Effects of bilateral intra-vlPAG administration of vehicle or morphine (1, 2.5, or 5 µg/200 nL/side) on c-Fos expression in the NRM in SD and WKY rats. Data are expressed as mean ± SEM, n = 5-7/group. *p<0.05 (SD-Morphine 1 µg or SD-Morphine 2.5 µg or SD-Morphine 5 µg vs SD-Vehicle), ^{+}p<0.05 (WKY-Morphine 5 µg vs WKY-Vehicle). Morph: morphine, NRM: nucleus raphe magnus, RVM: rostral ventromedial medulla, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

3.3.4.2. Dorsal horn of lumbar (L4-L6) spinal cord

Expression of c-Fos positive cells was measured in the superficial layers (laminae I and II) of the dorsal horn in the lumbar (L4-L6) segment of spinal cord (Figure 3.7A). Counting was performed in both ipsi- and contralateral sides with respect to formalin injection. KW test revealed a significant main effect ($\chi^2(15)=81.024$, ***p*<0.001**) on c-Fos expression in the DHSC. *Post hoc* tests showed that the c-Fos expression in all groups, irrespective of strain and drug treatment, was significantly higher on the ipsilateral side relative to the contralateral side (ipsilateral vs contralateral side, ***p*<0.01**; Figure 3.7C).

We further analysed the data splitting by side. In the ipsilateral side of DHSC (Figure 3.7D), two-way ANOVA revealed significant effects of strain ($F_{1,46}=25.342$, ***p*<0.001**) and treatment ($F_{3,46}=38.868$, ***p*<0.001**), but not of strain x treatment interaction ($F_{3,46}=2.186$, $p>0.05$), on c-Fos expression. *Post hoc* SNK analysis showed that bilateral intra-vlPAG administration of morphine at low dose (1 µg) significantly reduced c-Fos expression on the ipsilateral side in SD rats (SD-Morphine 1 µg vs SD-Vehicle, ***p*<0.05**); however, this effect was not seen in the WKY counterparts. High doses of morphine (2.5 and 5 µg) microinjected into the vlPAG resulted in a significant decrease in c-Fos expression on the ipsilateral side in both SD (SD-Morphine 2.5 µg or SD-Morphine 5 µg vs SD-Vehicle, ***p*<0.05**) and WKY (WKY-Morphine 2.5 µg or WKY-Morphine 5 µg vs WKY-Vehicle, ***p*<0.05**) rats. In the contralateral side of DHSC (Figure 3.7E), two-way ANOVA revealed a significant effect of treatment ($F_{3,46}=7.848$, ***p*<0.001**) but not of strain ($F_{1,46}=0.447$, $p>0.05$) or strain x treatment interaction ($F_{3,46}=0.521$, $p>0.05$). *Post hoc* analysis found that intra-vlPAG microinjection of morphine (1 and 2.5 µg) significantly reduced c-Fos expression on the contralateral side in SD rats (SD-Morphine 1 µg or SD-Morphine 2.5 µg vs SD-Vehicle, ***p*<0.05**). No such effect of morphine on the expression of c-Fos was observed in the contralateral side in WKY rats.


A)
I
II
III
I
V


B)
SD
WKY
contra
ipsi
contra
ipsi
Vehicle
Morph
1 µg
Morph
2.5 µg
Morph
5 µg
200 µm

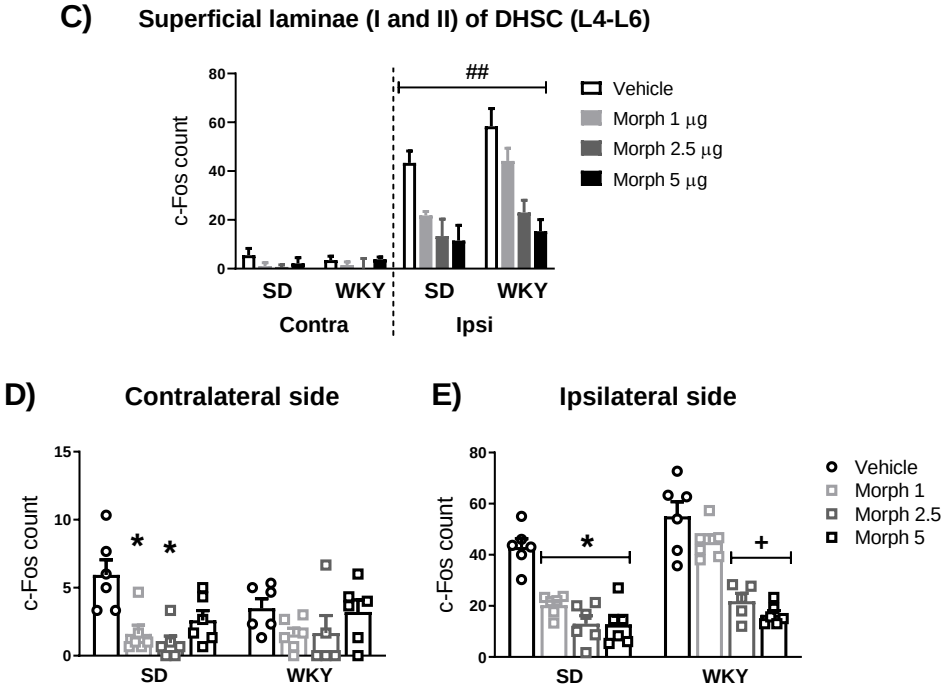


Figure 3.7: (A) Superficial laminae (I and II) of rat DHSC (open square) of lumber segment (L4-L6), adapted from Paxinos and Watson, 2007. (B) Representative images (10X magnification) of c-Fos expression in the superficial laminae of ipsi- and contralateral sides of DHSC (L4-L6) in SD (left panel) and WKY (right panel) rats receiving intra-vlPAG microinjection of vehicle or morphine. Grey arrows point out to some of the c-Fos positive cells in each section. Grey dotted area represents the area where c-Fos-like immunoreactive cells were counted. (C-E) Effects of bilateral intra-vlPAG administration of vehicle or morphine (1, 2.5, or 5 μg/200 nL/side) on c-Fos expression in the superficial laminae of ipsi- and contralateral sides of DHSC (L4-L6) in SD and WKY rats. Data are expressed as (C) median with interquartile range and (D and E) mean ± SEM, n = 5-6/group. *p<0.05 (SD-Morphine 1 μg or SD-Morphine 2.5 μg or SD-Morphine 5 μg vs SD-Vehicle), ^{+}p<0.05 (WKY-Morphine 2.5 μg or WKy-Morphine 5 μg vs WKY-Vehicle), $^{\#\#}p$<0.01 (ipsilateral vs contralateral side). C: contralateral, DHSC: dorsal horn of spinal cord, I: ipsilateral, Morph: morphine, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

3.3.5. Expression of MOP in the vlPAG in naïve WKY versus SD rats

No difference was found in the expression of MOP protein in the vlPAG between naive WKY and SD rats (left/contra: t_6= -1.266, p>0.05; right/ipsi: t_6= -1.351, p>0.05; Figure 3.8).

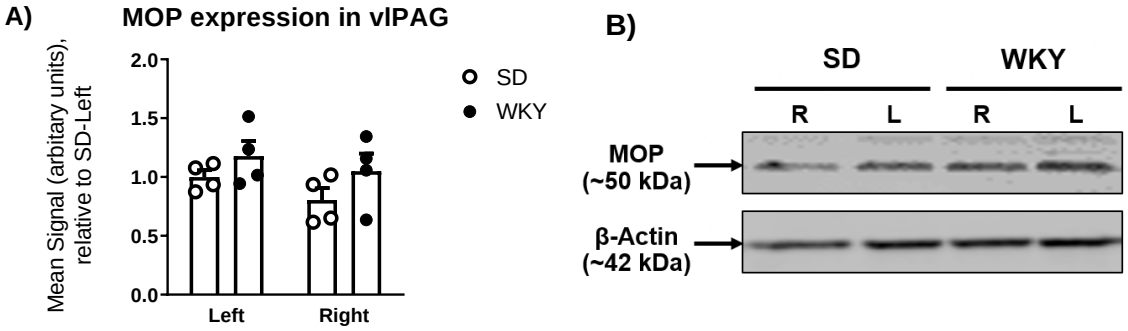


Figure 3.8: (A) Expression of MOP in the vlPAG in naïve SD and WKY rats. A separate cohort of rats of either strain was used to compare the expression of MOP protein in the left/contra and right/ipsi (with respect to formalin injection in the drug-treated cohort) vlPAG using Western immunoblotting. (B) Representative Western blot images showing the immunoreactive bands for MOP (~50 kDa) and β-actin (~42 kDa) in naïve SD and WKY rats. Data are expressed as mean ± S.E.M, n = 4/group. MOP: mu opioid receptor, SD: Sprague-Dawley, vlPAG: ventrolateral periaqueductal grey, WKY: Wistar-Kyoto.

3.4. Discussion

This chapter explored the effects of pharmacological modulation of MOP in the vlPAG on formalin-evoked nociceptive behaviour in SD and WKY rats. In the SD rats, intra-vlPAG administration of morphine at all three doses produced robust and prolonged antinociception. In comparison, intra-vlPAG administration of the high doses of morphine was antinociceptive in the WKY rats; the low dose of morphine injected into the vlPAG did not reduce formalin-evoked nociceptive behaviour in the WKY rats. Moreover, morphine-induced antinociceptive effect in both strains was associated with a concomitant increase in c-Fos expression in the NRM of RVM and a decrease in c-Fos expression on the ipsilateral side of dorsal horn of lumbar spinal cord. However, these changes in the expression of c-Fos in regions downstream of PAG were blunted in the WKY versus SD rats. In addition, MOP expression within vlPAG did not differ between naïve WKY and SD rats.

Similar to previous work in our laboratory (Burke et al., 2010; Rea et al., 2014; Madasu et al., 2016), WKY rats receiving an intra-vlPAG vehicle injection displayed higher formalin-evoked nociceptive behaviour compared to SD counterparts, confirming their hyperalgesic phenotype to noxious inflammatory stimulus. Direct injection of low dose of morphine into the vlPAG resulted in marked antinociception in the SD, but not in the WKY, rats, indicating hyporesponsivity to morphine-induced antinociception in the WKY rat strain. High doses of morphine administered into the vlPAG were antinociceptive in both strains. In agreement with previous studies (Hoffmann et al., 1998; Plesan et al., 1999; Terner et al., 2003; Hestehave et al., 2019b), we reported in Chapter 2 that WKY rats exhibited reduced responsivity to systemically administered morphine in tests employing acute (heat) noxious stimulus. Furthermore, we found that the WKY rats were hyporesponsive to morphine-induced antinociception in the formalin test, a model of persistent inflammatory noxious stimulus. The novel data presented herein suggest that the vlPAG is a key locus within the descending pain pathway for hyporesponsivity to morphine-induced antinociception in the WKY rats, compared to SD counterparts. As mentioned previously, the vlPAG contains a high density of MOP and approximately 36% of vlPAG neurons projecting to the RVM express MOP (Wang and Wessendorf, 2002). The MOP expressed in the vlPAG play a key role in the antinociceptive effects of systemically or centrally administered morphine (Rossi et al., 1994a; Commons et al., 2000; Lane et al., 2005; Bobeck et al., 2012). The basal

expression of the MOP protein in the vlPAG did not differ between the two strains. Thus, an alteration in the expression of MOP in the vlPAG seems unlikely to account for the differential antinociceptive potency of morphine in the WKY versus SD rats. Further investigation looking into potential strain-related alteration in MOP functionality in the vlPAG is warranted.

The PAG plays a prominent role in coping strategies to internal (e.g., pain) or external (e.g., threat) stressors. While activation of dlPAG/lPAG is mostly associated with the "fight or flight" response (active coping), activation of vlPAG results in quiescence and hyporeactivity (passive coping) (Keay and Bandler, 2001). Direct injection of morphine at doses ≥5 μg into the vlPAG induced immobility in rodents (Morgan et al., 1998; Morgan and Clayton, 2005), which was reversed by naloxone (Monassi et al., 1999; Miranda-Páez et al., 2016). In the current study, morphine at the doses employed did not have an overall effect on locomotor activity as measured by distance moved in the arena during pre- and post-formalin trials, suggesting morphine-induced antinociception was not merely due to an effect on motor activity. However, a significant reduction in grooming was observed in both strains following intra-vlPAG morphine injection (all three doses), particularly during post-formalin trial, which might indicate a vlPAG-mediated passive emotional coping strategy in response to an inescapable stress (e.g. deep somatic noxious stimulus) (Keay and Bandler, 1993, 2002). Another interesting observation was that a proportion of rats of both strains receiving high dose morphine into the vlPAG exhibited jumping response, a behaviour typically associated with aversion/escape. Activation of vlPAG has been mostly shown to mediate passive defensive behaviours. However, Morgan and Clayton (2005) reported that the vlPAG can mediate various stress-coping behaviours ranging from immobility to flight. Thus, the jumping response displayed by a proportion of rats and not by others in a given treatment group might suggest an inter-subject variability in terms of behavioural response to pharmacological manipulation.

The present study also examined whether a strain-related difference in the activation of descending inhibitory pain pathway following intra-vlPAG administration of morphine may account for the differential antinociceptive effects in WKY versus SD rats. Expression of c-Fos, a well characterised marker of neuronal activity, was measured in the NRM of RVM and in the superficial laminae of the dorsal horn of lumbar spinal cord, regions downstream of PAG. The RVM, an important relay station in the PAG-RVM-

spinal cord circuit within descending pain pathway, comprises of several nuclei of which the NRM predominantly receives descending projections from the ventrolateral subcolumn of PAG in rats as demonstrated in tract-tracing studies (Beitz et al., 1983; Cameron et al., 1995; Hudson and Lumb, 1996). Moreover, lesioning NRM abolished antinociceptive effect of morphine microinjected into the vlPAG, indicating vlPAG-NRM connectivity is critical in mediating morphine-induced antinociception (Young et al., 1984). We found that the reduction in formalin-evoked nociceptive behaviour after intra-vlPAG administration of morphine in the SD rats was associated with an increase in c-Fos expression in the NRM, compared to vehicle-treated counterparts. On the basis of responses to noxious cutaneous stimulation, RVM neurons are classified functionally into three distinct classes: ON, OFF, and NEUTRAL cells (Fields et al., 1983a; Heinricher et al., 1989). ON cells exhibit an increase in activity associated with nociceptive reflexes and are thought to facilitate nociception, while OFF cells show an abrupt decrease in ongoing discharge during noxious stimulation and are proposed to inhibit nociception. NEUTRAL cells are unaffected by noxious peripheral stimuli and their role in nociception is unclear. Systemic or direct injection of opioids (such as morphine, DAMGO, and DAGO) into the vlPAG suppresses ON cell and enhances OFF cell activities (with no effect on NEUTRAL cells) in the RVM, resulting in antinociception (Fields et al., 1983b; Barbaro et al., 1986; Cheng et al., 1986; Fang et al., 1989b; Heinricher et al., 1992b; McGaraughty et al., 1993; Rossi et al., 1994b; Tortorici and Morgan, 2002). Thus, increase in NRM c-Fos expression in the SD rats after intra-vlPAG morphine injection probably indicates an increase in OFF cell activity that is associated with antinociception. In addition, the blunted increase in morphine-induced NRM c-Fos expression in the WKY rats, compared to SD counterparts, might be due to a deficit of MOP-mediated alteration in ON and/or OFF cell activity in the RVM. Further electrophysiological studies are needed to fully elucidate any functional difference in the RVM neuronal populations in the two strains.

After intraplantar injection of formalin to the hindpaw in rats, c-Fos protein is rapidly expressed within an hour in the DHSC (Presley et al., 1990; Leah et al., 1992). Most of the nociceptive afferents originating from the periphery (hindpaw) terminate in the superficial laminae I and II of the dorsal horn in the lumbar (L4-L6) spinal segment (Swett and Woolf, 1985), resulting in the most intense expression of c-Fos in these superficial layers following a noxious stimulus. Opioids administered systemically or centrally

reduce formalin-evoked expression of c-Fos in the superficial layers of the spinal dorsal horn (Presley et al., 1990; Gogas et al., 1991, 1996), indicating opioid-mediated supraspinal modulation of nociceptive transmission via the spinal cord. In the present study, intra-vlPAG administration of morphine markedly suppressed c-Fos expression in the ipsilateral superficial layers of the dorsal horn in L4-L6 region of the spinal cord in SD rats. It is well established that RVM neurons including those originating from the NRM project primarily to the spinal dorsal horns (Fields et al., 1977, 1995; Basbaum et al., 1978; Cho and Basbaum, 1989) and modulate nociceptive transmission. Therefore, the reduction in c-Fos expression in the ipsilateral DHSC in the SD rats after intra-vlPAG morphine administration is likely to reflect the top-down modulation of spinal nociceptive transmission that is associated with antinociception. Similar to changes in c-Fos expression observed in the RVM, reduction in morphine-induced spinal c-Fos expression was blunted in the WKY rats, compared to SD counterparts, further suggesting a deficit in morphine-induced engagement of the top-down inhibition. Of note, though c-Fos expression was largely confined to the ipsilateral side, some Fos-like immunoreactive cells were expressed on the contralateral dorsal horn. Previous studies using unilateral noxious stimuli have reported expression of c-Fos on the opposite side of the spinal cord (Menétrey et al., 1989; Yamazaki et al., 2001), perhaps implying an increased sensitivity in these contralateral neurons. Also, stressful procedures (such as anaesthesia) induced contralateral c-Fos expression in rats after formalin injection (Quintero et al., 2003; Takasusuki et al., 2013), which might be another possible explanation of the results obtained here. However, we feel that the treatment effect on c-Fos expression on the contralateral side probably reflects the effect of bilateral injection of morphine into the vlPAG.

In conclusion, these findings provide further evidence for enhanced inflammatory nociceptive responding in the WKY rats. We identified, for the first time, the vlPAG as a key locus within the descending modulatory pain pathway for hyporesponsivity to the antinociceptive effects of morphine in the WKY versus SD rats. Moreover, the changes in the expression of c-Fos in the RVM and spinal cord correlate well with morphine-induced behavioural changes following noxious inflammatory stimulus in both SD and WKY rats. Thus, the strain-related differences in neuronal activity in regions downstream of vlPAG indicate a deficit in the MOP-induced engagement of the top-down nociception control system in the WKY rats that might underlie their hyperalgesic phenotype.

4. Chapter 4: Effects of modulating kappa-opioid receptors on the sensory and affective components of pain and anxiety/depression-related behaviours in Wistar-Kyoto and Sprague-Dawley rats

4.1. Introduction

Opioids are one of the most widely used analgesic classes for the management of pain. Most of the clinically used opioids act primarily through the MOP and are commonly indicated in the management of both acute and chronic pain. However, their utility has come under scrutiny recently owing to several well-described MOP-mediated side effects including nausea, vomiting, constipation, respiratory depression, sedation, euphoria, tolerance, dependence, and addiction (Holden et al., 2005; Wang et al., 2019). Hence, the need to develop efficacious analgesics without the adverse effects typically associated with MOP agonists remains a high priority. In this regard, other opioid receptors are currently being investigated as putative drug targets for pain and associated comorbid conditions.

A promising alternative target is the kappa opioid system. The kappa opioid receptors (KOP) are widely distributed in both central and peripheral tissues (Minami et al., 1993; Depaoli et al., 1994; Gutstein et al., 1998; Peckys and Landwehrmeyer, 1999; Snyder et al., 2018), particularly in several key brain regions with overlapping functions including modulation of pain, stress response, and affect. Activation of KOP results in antinociception (Kivell and Prisinzano, 2010). In addition, pharmacological studies in transgenic mice lacking KOP or prodynorphin [PDYN, precursor of the endogenous KOP preferential peptide dynorphin (Chavkin et al., 1982)] suggest a key role of the KOP system in mediating antinociception upon exposure to noxious visceral, inflammatory, and thermal stimuli (Simonin et al., 1998; Wang et al., 2001). However, unlike other classical opioid receptors (MOP and DOP), KOP activation in the brain induces negative affective states, possibly by modulating monoaminergic neurotransmission (Tao and Auerbach, 2005; Berger et al., 2006; Kreibich et al., 2008; Land et al., 2009; Chefer et al., 2013; Tejeda et al., 2013). For instance, clinical studies showed that synthetic and naturally occurring KOP agonists cause dysphoria (a profound unpleasant or aversive

state) and elicit anxiogenic and psychotomimetic effects in humans (Pfeiffer et al., 1986; Rimoy et al., 1994; Walsh et al., 2001; González et al., 2006). Moreover, systemic or site-specific administration of KOP agonists produces conditioned place aversion (Shippenberg and Herz, 1987; Bals-Kubik et al., 1993; Sante et al., 2000; Zhang et al., 2005b; Bruchas et al., 2007; Land et al., 2008; Tejeda et al., 2013; Anderson et al., 2014; Robles et al., 2014), anhedonia (Todtenkopf et al., 2004), and increases immobility in the forced swim test (Mague et al., 2003; Carlezon et al., 2006) in rodents. It should be noted that although these centrally mediated side effects of selective KOP agonists limit their development as analgesics, recent clinical trials suggest peripherally restricted KOP agonists are effective as well, offering a more favourable side effect profile while retaining antinociceptive efficacy (Albert-Vartanian et al., 2016).

Deletion of genes encoding KOP or PDYN reduces the aversive and prodepressive-like behaviours induced via KOP activation in mice (Simonin et al., 1998; McLaughlin et al., 2003; Land et al., 2009; Wittmann et al., 2009; Kastenberger et al., 2012; Chefer et al., 2013). In addition, pharmacological blockade of KOP produces antidepressant-like and anxiolytic effects in a number of rodent models (Mague et al., 2003; Beardsley et al., 2005; Knoll et al., 2007, 2011; Zhang et al., 2007; Wittmann et al., 2009; Carr et al., 2010; Rorick-Kehn et al., 2014; Browne et al., 2015) (for review see Hang et al., 2015; Carlezon and Krystal, 2016). These studies clearly indicate that the KOP system is a key biological substrate in mediating negative affect. Thus, the KOP antagonists represent another potential class of therapeutics for the treatment of neuropsychiatric diseases including anxiety and depression and are currently under active investigation in clinical trials (Ehrich et al., 2015; Reed et al., 2018).

Growing evidence indicates that the KOP system contributes to the affective (emotional) response associated with pain and stress (for review see Knoll and Carlezon, 2010; Cahill et al., 2014; Margolis and Karkhanis, 2019; Tejeda and Bonci, 2019). The expression and function of KOP and dynorphin are upregulated in several brain regions that modulate affective responding both in chronic pain (Liu et al., 2019b; Massaly et al., 2019) and following exposure to stress (Shirayama et al., 2004; Bruchas et al., 2007; Land et al., 2008) in rodents. In addition, pain-induced tonic-aversion and anhedonia are alleviated by KOP blockade (specifically in the amygdala and nucleus accumbens, respectively) or selective ablation of KOP in midbrain dopaminergic neurons (Liu et al., 2019b; Massaly et al., 2019; Navratilova et al., 2019). Similarly, depressive-like behaviours induced by

chronic stress are blocked by either directly inhibiting KOP, disrupting PDYN gene, or interfering with KOP signalling (Newton et al., 2002; McLaughlin et al., 2003; Shirayama et al., 2004; Bruchas et al., 2007; Land et al., 2008; Lemos et al., 2012). Thus, attenuation of aversive aspects of ongoing pain by KOP antagonists may represent a novel therapeutic strategy for comorbid pain-negative affect (Xie et al., 2017; Navratilova et al., 2019).

The involvement of KOP in mediating hyperalgesia associated with negative affect has yet to be determined. However, increased [^{35}S]GTPγS binding of KOP-selective ligands in the amygdala has been described in rat models of chronic pain exhibiting concomitant anxiety-like behaviour, suggesting an augmented functionality of amygdalar KOP in comorbid anxiety and pain (Narita et al., 2006a). Constitutive KOP or PDYN knockout mice display enhanced mechanical allodynia but reduced pain-associated anxiety-like behaviour in a model of osteoarthritis, suggesting a complex modulatory role of the KOP in comorbid pain-negative affect conditions (Negrete et al., 2017). Previous studies also suggest that an increased activity of the KOP system may contribute to the behavioural phenotype of the WKY rat strain. Microarray analysis has revealed higher expression of KOP-encoding gene in the locus coeruleus, an important source of descending noradrenergic regulation of pain, in WKY versus SD rats (Pearson et al., 2006). WKY rats also exhibit higher expression of KOP and dynorphin in the amygdala, nucleus accumbens, and piriform cortex (regions implicated in nociception, stress and affect), compared to SD rats (Carr et al., 2010; Dennis et al., 2016; Burke et al., 2019). Furthermore, KOP antagonists produce antidepressant-like behavioural effects in the forced swim test in WKY, but not in SD, rats (Carr et al., 2010; Browne et al., 2015; Burke et al., 2019). Taken together, these studies suggest that the WKY rats could be more sensitive to modulation of the KOP system than SD counterparts.

Therefore, this chapter examines the hypothesis that an altered expression and/or functionality of the KOP system may underlie hyperalgesic phenotype in the WKY rats, compared to SD counterparts. Specific aims of the experiments described in this chapter were as follows:

- To compare the effects of systemic administration of the KOP agonist U50488 (Lahti et al., 1982) and the KOP antagonist DIPPA (Chang et al., 1994b) on nociceptive responding to acute (thermal) and persistent (tonic inflammatory) noxious stimuli in WKY versus SD rats.

- To investigate the effects of systemic administration of U50488 and DIPPA on the affective component of persistent inflammatory pain in a place conditioning paradigm in WKY and SD rats.
- To compare the effects of systemic administration of U50488 and DIPPA on anxiety- and depression-like behaviours in WKY versus SD rats.
- To determine the expression of the genes encoding KOP and PDYN in discrete brain regions involved in processing pain and negative affect in WKY and SD rats.

4.2. Materials and methods

4.2.1. Animals

Male WKY and SD rats (8-10 weeks old, 180-225 g on arrival; Envigo, Bicester, UK) were used in this study. Rats were maintained under a standard 12:12 h light/dark cycle (lights on from 07:00-19:00 h) in a temperature- (21±2°C) and humidity-controlled (45-55%) room throughout the study. Upon arrival, all animals were housed in groups of 3-4 per cage in plastic bottom cages (45x20x20 cm^3) containing 3Rs basic bedding (>99% recycled paper; 3Rs Lab, Fibrecycle Ltd, North Lincolnshire, UK) and sizzle nest material (LBS Biotechnology, Horley, UK) to acclimatise to the unit. Animals were housed singly in cages for 7 days prior to commencing behavioural testing. All experimental procedures were carried out during the light phase between 08:00 and 18:00 h. Food (14% protein rodent diet; Envigo, Bicester, UK) and water were available *ad libitum*. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway. The work was carried out under license from the Health Products Regulatory Authority in the Republic of Ireland and in accordance with EU Directive 2010/63. The study was also designed and carried out in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

4.2.2. Chemicals and drug preparation

The KOP agonist U50488 hydrochloride [trans-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride; Tocris, Bristol, UK] was dissolved in sterile 0.89% w/v NaCl (Fisher Scientific, Leicestershire, UK). The KOP antagonist DIPPA hydrochloride [2-(3,4-Dichlorophenyl)-N-methyl-N-{(1S)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl}acetamide hydrochloride; Tocris, Bristol, UK] was dissolved in 20% dimethyl sulphoxide (DMSO; Sigma-Aldrich, Wicklow, Ireland) in sterile deionised water. The final doses of the drugs were corrected for the salt form and given as mg free base equivalent per kg body weight. All drugs were freshly prepared on the days of behavioural testing and administered via the subcutaneous (s.c.) route in an injection volume of 1 mg/mL. For Experiment 2, formalin (Sigma-Aldrich, Wicklow, Ireland) was diluted from a stock of 37% to 2.5% in sterile 0.89% w/v NaCl on the test day.

4.2.3. Study design

The study consisted of two separate experiments (Figure 4.1). Animals were randomly assigned to treatment groups in Experiment 1 and subsequently reallocated into treatment groups for Experiment 2 following a within-subjects modified Latin square design to ensure counterbalancing of treatments over the course of the study (see Table 4.1 for experimental groups and n numbers). In both experiments, each animal received two injections to control for the vehicles used and times of drug administration. The sequence of treatments was randomised in all experiments to control for the order of testing and time of the day. A period of 7 days was allowed for washout of the drugs between each experiment.

Experiment 1 compared dose responses of U50488 (1, 2.5, and 5 mg/kg, s.c.) and DIPPA (2.5 and 5 mg/kg, s.c.) on nociceptive responding to noxious heat stimulus and anxiety- and depression-like behaviours in WKY and SD rats. The doses and time points of administration of U50488 and DIPPA were chosen based on evidence from the literature demonstrating *in vivo* efficacy in rat models of pain and/or negative affect (Millan et al., 1987; Bianchi and Panerai, 1993; Privette and Terrian, 1995; Terner et al., 2005; Gallantine and Meert, 2008; Carr and Lucki, 2010; Carr et al., 2010). Animals were injected with DIPPA or its vehicle (20% DMSO) 24 h before testing. This time point of administration of DIPPA was chosen based on a previous study showing KOP agonist-like activity of DIPPA within the first 4 h of administration but significant KOP antagonist-like activity later (without agonist-like effects) for up to 48 h (Chang et al., 1994b). On the test day, following administration of U50488 or its vehicle (0.89% NaCl), rats were tested on the hot plate (at +15 and +30 min), followed 5 min later by exposure to the EPM for 5 min, then in the OF for another 5 min and subsequently in the FST for a period of 15 min.

Experiment 2 assessed the effects of U50488 (1 and 2.5 mg/kg, s.c.) and DIPPA (2.5 mg/kg, s.c.), alone or in combination, on the sensory and affective components of pain in the formalin-induced conditioned pain aversion (F-CPA) paradigm in WKY and SD rats. The doses of U50488 and DIPPA used in Experiment 2 were based on the results of Experiment 1. The F-CPA paradigm consists of 3 sessions: preconditioning, conditioning, and postconditioning, which are described in detail later in section 4.2.4.5. Briefly, rats were allowed to explore freely both compartments on the preconditioning day. On conditioning day 1 (non-formalin day), rats were randomly confined to a compartment

for 60 min, immediately after which they were administered with DIPPA or 20% DMSO and were returned to their home cages. 23 h later on conditioning day 2 (formalin test day), all rats received an intraplantar formalin injection into the right hindpaw under brief isoflurane anaesthesia. U50488 or 0.89% NaCl was administered 15 min before formalin injection. Following formalin injection, animals were confined to the other compartment (opposite to that of conditioning day 1) for 60 min. On postconditioning day, rats were again allowed to freely access both compartments after which they were immediately euthanised by decapitation.

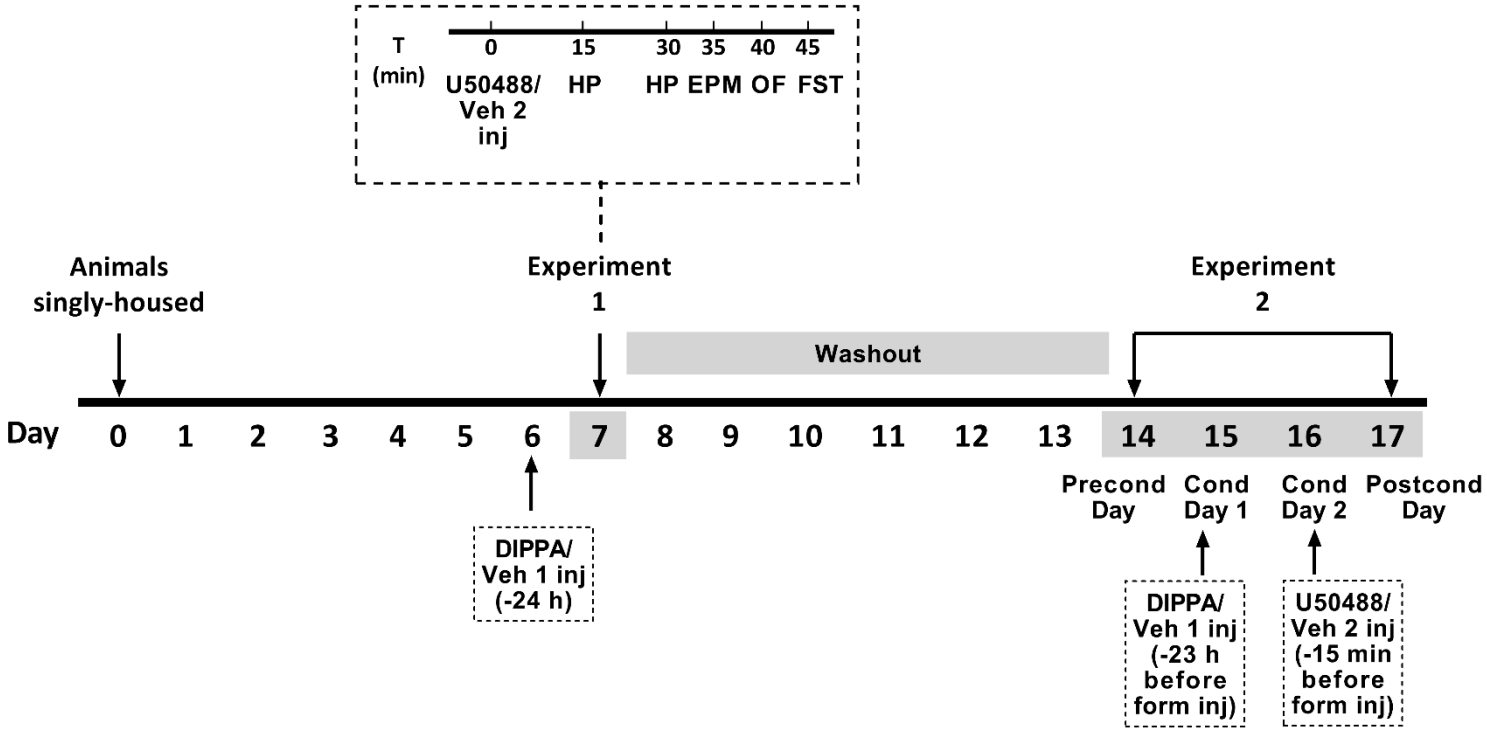


Figure 4.1: Schematic of the study outlining the design, timeline and behavioural tests of Experiments 1 and 2. A period of 7 days was allowed for washout of the drugs between each experiment. Time points of drug administration are relative to behavioural testing. Cond: conditioning, EPM: elevated plus maze, form: formalin, FST: forced swim test, HP: hot plate, inj: injection, Precond: preconditioning, Postcond: postconditioning, OF: open field, T: time, Veh 1: 20% DMSO (vehicle for DIPPA), Veh 2: 0.89% NaCl (vehicle for U50488).

Table 4.1: Summary of experimental groups for Experiments 1 and 2. n: number, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Group	Strain	Treatment, s.c.	n
Experiment 1			
1	SD	Vehicle	8
2	SD	U50488 1 mg/kg	8
3	SD	U50488 2.5 mg/kg	8
4	SD	U50488 5 mg/kg	8
5	SD	DIPPA 2.5 mg/kg	8
6	SD	DIPPA 5 mg/kg	8
7	WKY	Vehicle	8
8	WKY	U50488 1 mg/kg	8
9	WKY	U50488 2.5 mg/kg	8
10	WKY	U50488 5 mg/kg	8
11	WKY	DIPPA 2.5 mg/kg	8
12	WKY	DIPPA 5 mg/kg	8
Experiment 2			
1	SD	Vehicle	8
2	SD	U50488 1 mg/kg	8
3	SD	U50488 2.5 mg/kg	8
4	SD	U50488 1 mg/kg + DIPPA 2.5 mg/kg	8
5	SD	U50488 2.5 mg/kg + DIPPA 2.5 mg/kg	8
6	SD	DIPPA 2.5 mg/kg	8
7	WKY	Vehicle	8
8	WKY	U50488 1 mg/kg	8
9	WKY	U50488 2.5 mg/kg	8
10	WKY	U50488 1 mg/kg + DIPPA 2.5 mg/kg	8
11	WKY	U50488 2.5 mg/kg + DIPPA 2.5 mg/kg	8
12	WKY	DIPPA 2.5 mg/kg	8

4.2.4. Behavioural testing

4.2.4.1. Hot plate test

To assess nociceptive responding to an acute noxious heat stimulus, the hot plate test was carried out as described in Chapter 2 (see section 2.2.4.1.1). Baseline latency to respond on the hot plate was measured once in WKY and SD rats. Following baseline testing, DIPPA or 20% DMSO was administered 24 h before testing. On the test day (in Experiment 1), animals were administered with U50488 or 0.89% NaCl and tested again on the hot plate at two time points: +15 and +30 min, relative to U50488 or 0.89% NaCl administration.

4.2.4.2. Elevated plus maze test

The effects of U50488 or DIPPA on anxiety-related behaviours in WKY and SD rats were assessed in the EPM test as described in Chapter 2 (see section 2.2.4.2.1).

4.2.4.3. Open field test

Following EPM test, rats were immediately exposed to the OF test to compare the effects of U50488 or DIPPA on locomotor and anxiety-related behaviours in WKY and SD rats. The test was carried out as described previously in Chapter 2 (see section 2.2.4.2.2). The exception was that the floor of the circular arena used in this study was made up of reflective aluminium sheet.

4.2.4.4. Forced swim test

The effects of U50488 or DIPPA on behavioural despair in the WKY and SD rats were assessed using a single exposure to the modified FST as described in Chapter 2 (see section 2.2.4.2.3).

4.2.4.5. Formalin-induced conditioned place aversion paradigm

The F-CPA test combines the tonic persistent pain model, the formalin test, with the place-conditioning paradigm enabling simultaneous measurement of behaviours that reflect both the sensory and aversive/affective components of the noxious (inflammatory) stimulus. In the current study, F-CPA was conducted by using a counterbalanced, unbiased design as described previously (Johansen et al., 2001; Lei et al., 2004) with slight modifications. The place-conditioning apparatus (Figure 4.2) consisted of a wooden arena (64x31x32 cm^3) divided into two compartments (30 lux) by a central partition with

a small opening, allowing the animal to move freely between the two sides. Each compartment was characterised by distinct visual and olfactory stimuli, which was based on published work (Johansen et al., 2001). One conditioning compartment had horizontal (black and white) stripes on the walls and an odour of 1% acetic acid (Sigma-Aldrich, Wicklow, Ireland), whereas the other had vertical stripes and a cinnamon scent (Nature's Answer®, NY, USA) associated with it. In each compartment the olfactory cue was applied by dipping a cotton bud in acetic acid/cinnamon and securing it to the top portion of the wall opposite to the central partition. The arena was placed on a clear Perspex top and a video camera located underneath the arena was used to record the behaviours during the 4-day sessions. The arena was cleaned between animals with warm soapy water and then thoroughly with clean water to remove any remaining odour.

On the preconditioning day, animals were placed randomly (counterbalancing within a treatment group) into one of the compartments, facing the central opening and allowed unrestricted movement to both compartments for 20 min. Distance moved (cm) and time (s) spent in each compartment were recorded and analysed later using the EthoVision software (EthoVision XT, version 11.5; Noldus, Wageningen, Netherlands). On conditioning day 1, animals did not receive any noxious stimulus and were randomly confined to one of the conditioning compartments for 60 min, immediately after which they received a subcutaneous injection of DIPPA or 20% DMSO (23 h before formalin injection) and were returned to their home cages. On conditioning day 2, all animals received an intraplantar formalin injection (2.5%, 50 µL) into the right hindpaw under brief isoflurane anaesthesia (3% in 0.8 L/min O_2). U50488 or 0.89% NaCl was administered subcutaneously 15 min before formalin injection. Immediately after formalin injection, rats were placed into the other conditioning compartment (opposite to that of conditioning day 1). The test began when the rat righted itself and lasted for 60 min during which the animal was confined to the formalin-paired compartment. Nociceptive and exploratory (distance moved, rearing, grooming, and defaecation) behaviours during formalin trial were recorded and analysed later with EthoVision in an identical manner described in Chapter 2 (see section 2.2.4.1.2). The compartments paired with formalin injections were counterbalanced within a treatment group. On the postconditioning day, animals were placed into the arena in the same compartment as the preconditioning day and allowed to move freely to both compartments for 20 min. Distance moved and time spent in each compartment were recorded. A CPA score was

calculated for each rat as the difference in time spent in the formalin-paired compartment on the pre- and postconditioning sessions. A negative score denotes CPA, i.e., a decrease in time spent in the formalin-paired compartment on the postconditioning day relative to the preconditioning day. Formalin-induced oedema was also assessed by measuring the diameter of the formalin-injected hindpaw with Vernier callipers immediately before injecting formalin (on conditioning day 2) and after euthanasia (on postconditioning day).

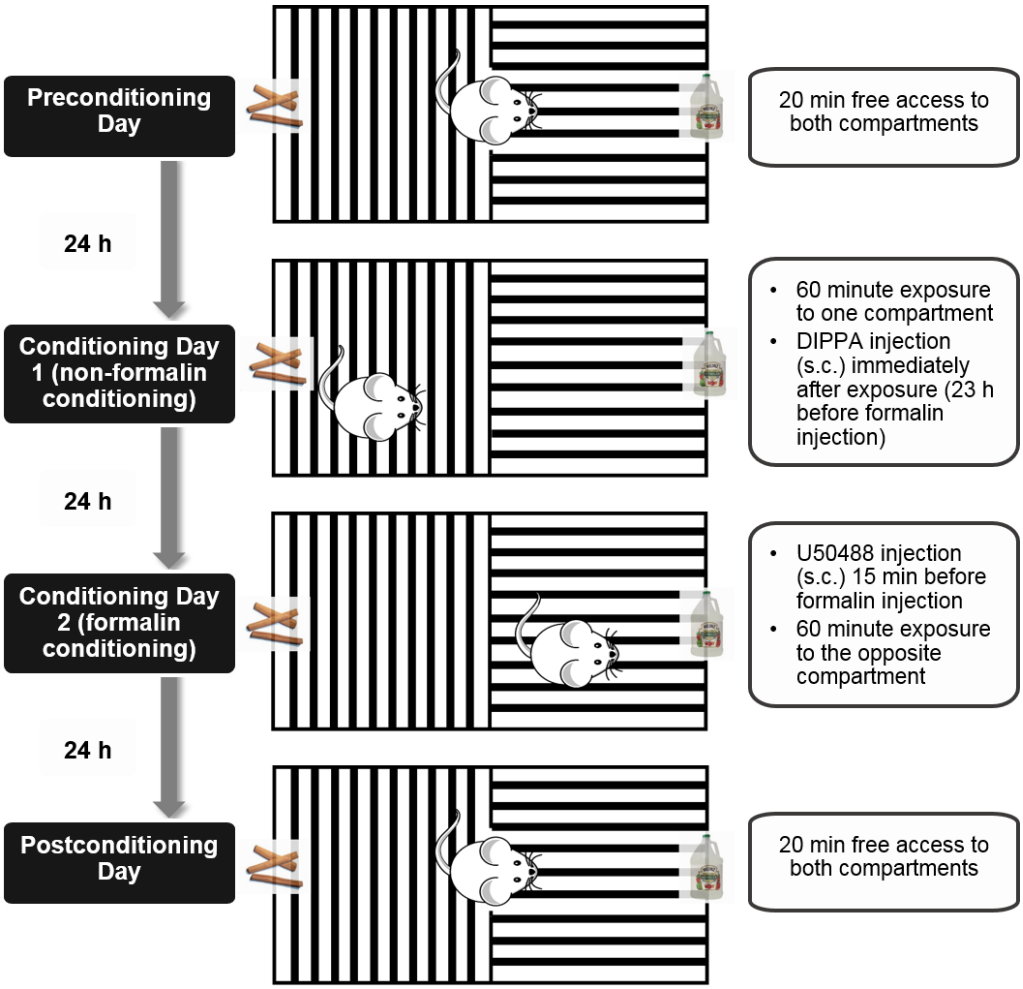


Figure 4.2: Schematic of F-CPA apparatus and behavioural testing timeline. F-CPA: formalin-induced conditioned place aversion, s.c.: subcutaneous.

4.2.5. Real-time quantitative polymerase chain reaction (RT-qPCR)

The brain tissues obtained from the naïve cohort of rats to analyse the expression of genes encoding MOP and POMC in Chapter 2 were used in this chapter to compare the basal expression of the genes encoding KOP and PDYN in WKY and SD rats. RT-qPCR was performed in an identical manner as described previously in Chapter 2 except for the target primers (*Oprk1* and *Pdyn*; Bio-Sciences, Dublin, Ireland) used, which are listed below (Table 4.2):

Table 4.2: Assay IDs and fluorescent labels for the target and endogenous control genes examined in rat brain tissues by RT-qPCR.

Target gene	Assay ID	Fluorescent label	Control gene	Assay ID	Fluorescent label
KOP (*Oprk1)*	Rn00567737_m1	FAM	β-actin (*Actb*)	Rn00667869_m1	VIC
PDYN (*Pdyn)*	Rn00571351_m1	FAM			

4.2.6. Statistical analysis

The SPSS statistical software (IBM SPSS Statistics, version 24 for Windows; SPSS Inc, Chicago, IL, USA) was used to analyse all data. In all datasets the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was considered an outlier and excluded from subsequent analysis. The normality and homogeneity of variance of the datasets were checked using Shapiro-Wilk and Levene's tests, respectively. The timecourse behavioural data were analysed with repeated measures analysis of variance (RM ANOVA) with strain and drug treatment as between-subject factors and time as within-subject factor. For RM ANOVA, sphericity of the datasets was checked with Mauchly's Test for Sphericity; if the assumption of sphericity was violated, a Greenhouse-Geisser correction was used. Other behavioural data except for defaecation were analysed using two-way ANOVA with strain and drug treatment as factors. Initial preference for a compartment on preconditioning day in F-CPA was analysed in each strain using two-way ANOVA with side and drug treatment as factors.

Post hoc Student-Newman-Keuls (SNK) test was carried out for pairwise group comparisons, where appropriate. KOP and PDYN gene expression data in naïve rats were analysed using Student's unpaired, two-tailed t-test. The level of significance was deemed $p<0.05$. If the data were not normally distributed and/or the variance was not homogeneous, three transformations were applied, in the order: square root, natural logarithm, and ranking of the data values to evaluate if parametric statistics can be used. In addition, if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular dataset being analysed, parametric statistical approach was still employed (Moore et al., 2009).

If any dataset was ordinal (e.g. defaecation) or found to be not normally distributed and/or the variance was not homogeneous even after transformation, non-parametric statistical analysis was performed using Kruskal-Wallis (KW) one-way analysis of variance by rank, followed by *post hoc* Mann-Whitney U-test (*p*-value was adjusted using Bonferroni-Holm correction for multiple comparisons), where appropriate. In all cases statistical analysis of a dataset was carried out for all the groups together. However, in some instances, the groups are graphed separately to present the results clearly in a reader-friendly format. Data are expressed as either mean ± standard error of the mean (S.E.M) or median with interquartile range (IQR) depending on the statistical approach undertaken, parametric or non-parametric, respectively.

4.3. Results

4.3.1. Experiment 1

4.3.1.1. Effects of U50488 and DIPPA nociceptive responding to noxious thermal stimulus in WKY and SD rats

The hot plate test was used to assess the dose responses of U50488 (1, 2.5, and 5 mg/kg, s.c.) and DIPPA (2.5 and 5 mg/kg, s.c.) on nociceptive responding to acute noxious heat in SD (Figure 4.3A) and WKY (Figure 4.3B) rats. At baseline, latency to respond on the hot plate did not differ between WKY and SD rats, as was the case in Chapter 2. Rats were tested on the hot plate again at +15 and +30 min following administration of U50488 or its vehicle. Two-way RM ANOVA revealed significant effects of time ($F_{1.807,137.299}$=102.914, ***p*<0.001**), time x strain ($F_{1.807,137.299}$=3.805, ***p*<0.05**), and time x treatment ($F_{9.033,137.299}$=3.549, ***p*<0.01**) in tests of within-subjects effects. A significant overall effect of treatment ($F_{5,76}$=8.088, ***p*<0.001**) was also found in tests of between-subjects effects on response latency. Further *post hoc* SNK analysis showed that, 15 min post-drug administration, U50488 dose-dependently increased response latency on the hot plate in both SD and WKY rats, compared to their respective vehicle counterparts. However, at the +30 min time point, U50488 significantly increases response latency in the SD rats (SD-U50488 2.5 mg/kg or SD-U50488 5 mg/kg vs SD-Vehicle, ***p*<0.05**); this effect failed to reach statistical significance in the WKY counterparts.

Interestingly, at the +15 min time point, DIPPA (5 mg/kg) significantly increased latency to respond on the hot plate in SD rats (SD-DIPPA 5 mg/kg vs SD-Vehicle, ***p*<0.05**). Similarly, a trend for DIPPA-induced antinociceptive effect was observed in the WKY counterparts (WKY-DIPPA 5 mg/kg vs WKY-Vehicle, p=0.079), which failed to reach statistical significance.

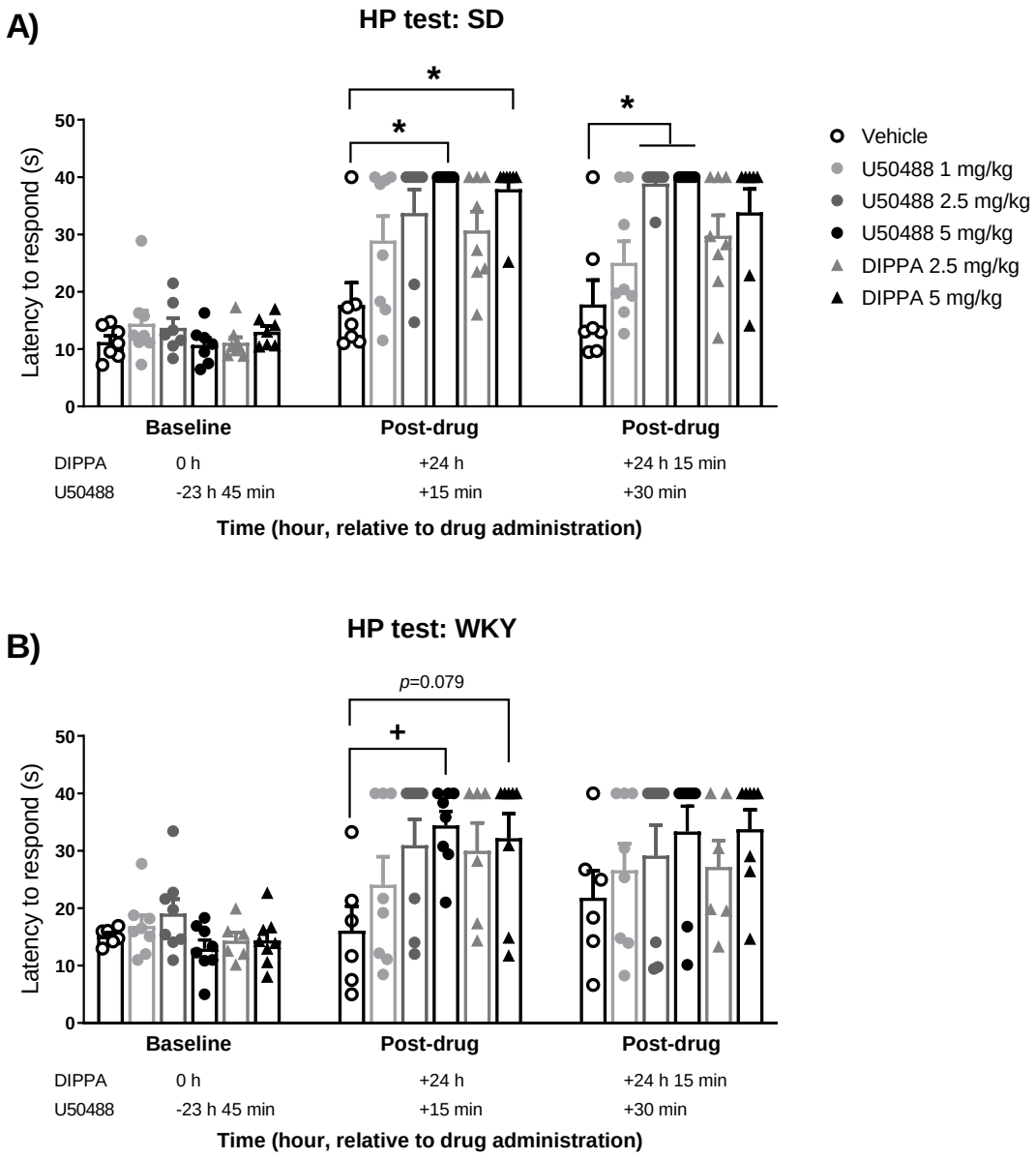


Figure 4.3: Effects of U50488 (1, 2.5, and 5 mg/kg, s.c.) and DIPPA (2.5 and 5 mg/kg, s.c.) on nociceptive responding to acute noxious heat stimulus (Experiment 1) in (A) SD and (B) WKY rats. X-axis shows the time points of testing relative to administration of each drug. Data are expressed as mean ± SEM, n = 6-8/group. *p<0.05 (SD-U50488 2.5 mg/kg or SD-U50488 5 mg/kg vs SD-Vehicle), ^{+}p<0.05 (WKY-U50488 5 mg/kg vs WKY-Vehicle). HP: hot plate, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

4.3.1.2. Effects of U50488 and DIPPA on anxiety-related behaviour and general locomotor activity in WKY and SD rats

After the hot plate test, animals were exposed to the EPM and then immediately to the OF to assess the effects of U50488 and DIPPA on anxiety-like behaviours and general locomotor activity. As mentioned previously in Chapter 2, activity in the open arms in EPM is related to anxiety-like behaviours while activity in the closed arms can be used as an indirect measure of locomotor activity. Two-way ANOVA revealed significant effects of treatment ($F_{5,95}$=3.510, ***p*<0.01**) and strain x treatment interaction ($F_{5,95}$=3.400, ***p*<0.01**), but not of strain ($F_{1,95}$=1.002, p>0.05), on time spent in open arms (Figure 4.4A). Further *post hoc* test showed that there were no significant differences between drug-treated groups within each strain or between the two strains. KW test revealed a significant main effect ($\chi^2(11)$=20.5980, ***p*<0.05**) on number of entries into open arms but no significant difference between relevant groups was found in *post hoc* tests (Figure 4.4B). Two-way ANOVA showed significant effects of strain ($F_{1,95}$=30.940, ***p*<0.001**) and strain x treatment interaction ($F_{5,95}$=4.241, ***p*<0.01**), but not of treatment ($F_{5,95}$=2.194, p>0.05), on time spent in closed arms (Figure 4.4C). *Post hoc* test revealed that high doses of U50488 (2.5 and 5 mg/kg) significantly increased time spent in closed arms in the SD rats (SD-U50488 2.5 mg/kg or SD-U50488 5 mg/kg vs SD-Vehicle, ***p*<0.05**), but not in the WKY counterparts. A significant main effect ($\chi^2(11)$=56.572, ***p*<0.001**) on number of entries into closed arms was observed in KW test (Figure 4.4D). Further *post hoc* analysis revealed that vehicle-treated WKY rats exhibited a significant reduction in closed arm entries, compared to SD counterparts (WKY-Vehicle vs SD-Vehicle, ***p*<0.001**), indicating their hypolocomotor trait. No significant effect of U50488 was found on number of entries into closed arms in SD or WKY rats. In addition, DIPPA at the doses tested (2.5 and 5 mg/kg) did not affect closed arm activities in either strain.

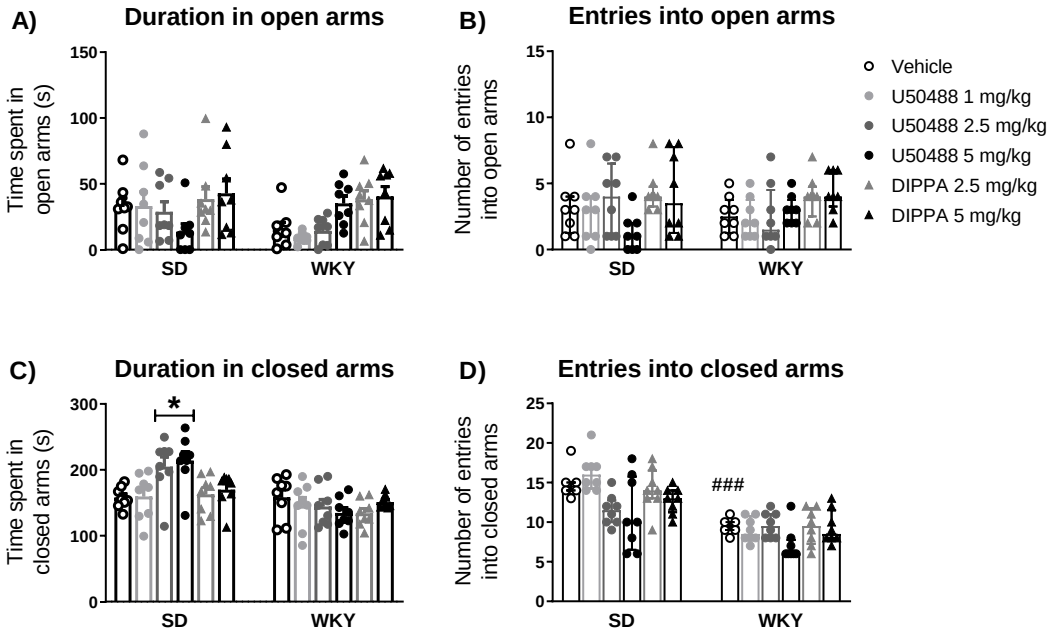


Figure 4.4: Effects of U50488 (1, 2.5, and 5 mg/kg, s.c.) and DIPPA (2.5 and 5 mg/kg, s.c.) on duration and number of entries in (A and B) open and (C and D) closed arms in the EPM test in SD and WKY rats (Experiment 1). Data are expressed as (A and C) mean ± SEM and (B and D) median with IQR, n = 8/group. *p<0.05 (SD-U50488 2.5 mg/kg or SD-U50488 5 mg/kg vs SD-Vehicle), $^{###}p$<0.001 (WKY vs SD counterpart). EPM: elevated plus maze, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

In the OF test, general locomotor activity was measured by distance moved in the arena (Figure 4.5A). KW test revealed a significant main effect ($\chi^2(11)=50.275$, ***p*<0.001**) on the distance moved. *Post hoc* analysis showed vehicle-treated WKY rats exhibited a significant reduction in distance moved compared to SD controls (WKY-Vehicle vs SD-Vehicle, ***p*<0.05**), indicating their hypolocomotor phenotype. Systemic injection of U50488 or DIPPA at the doses administered did not affect distance moved in SD or WKY rats. KW test showed a significant main effect ($\chi^2(11)=34.425$, ***p*<0.001**) on pellets excreted during the 5 min trial (Figure 4.5B) but further *post hoc* analysis revealed that there was no significant difference between relevant groups. Also, there were no significant main effects on centre zone activities in KW analysis [number of entries into centre zone: $\chi^2(11)=12.869$, $p>0.05$; time spent in centre zone: $\chi^2(11)=7.419$, $p>0.05$], which were indices for anxiety-related behaviour in the OF test (Figure 4.5C and 4.5D).

Of note, a proportion of the WKY rats (12 out of 48 rats or 25%) spent a long time (>100 s) in the centre zone of the OF. There was no significant main effect ($\chi^2(11)=19.477$, $p>0.05$) on latency to leave the centre zone in KW test (Figure 4.5E) .

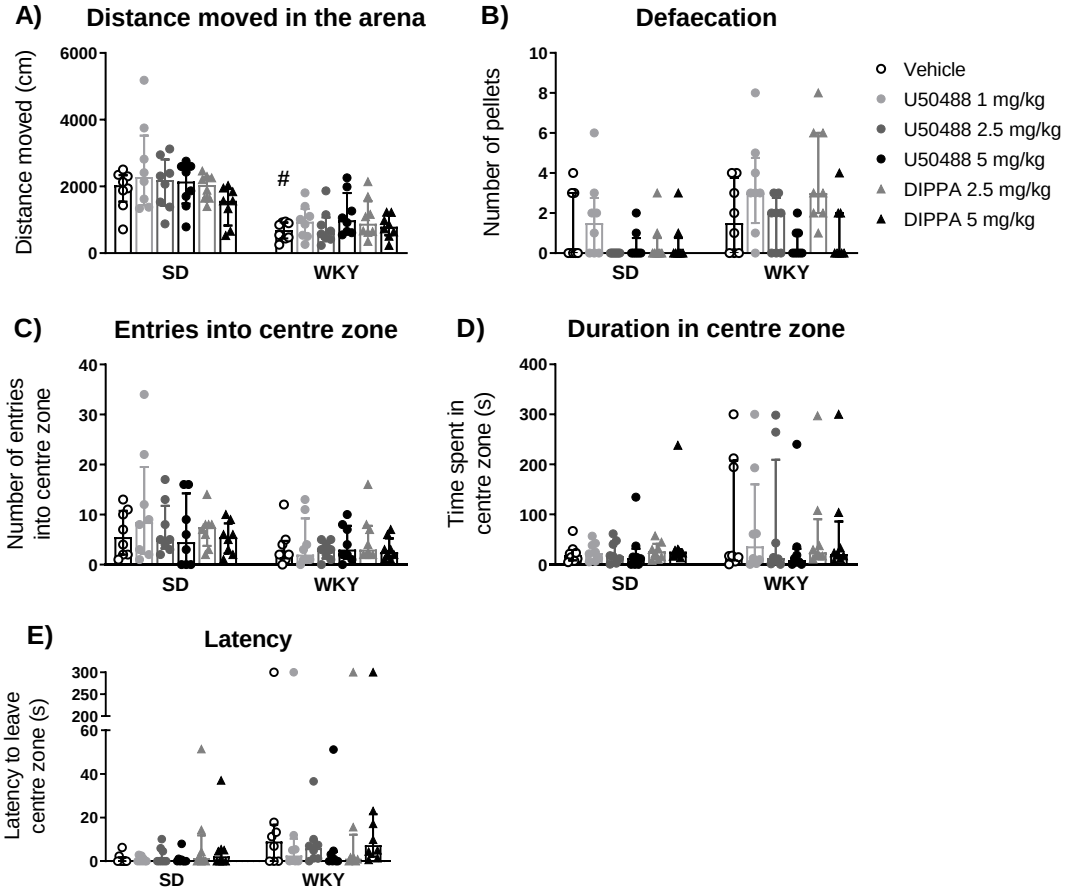


Figure 4.5: Effects of U50488 (1, 2.5, and 5 mg/kg, s.c.) and DIPPA (2.5 and 5 mg/kg, s.c.) on (A) distance moved in the arena, (B) defaecation (number of pellets produced), (C) number of entries into centre zone, (D) time spent in centre zone, and (E) latency to leave centre zone in the OF test in SD and WKY rats (Experiment 1). Data are expressed as median with IQR, n = 8/group. $^{\#}p<0.05$ (WKY vs SD counterpart). OF: open field, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

4.3.1.3. Effects of U50488 and DIPPA on behaviours in the FST in WKY and SD rats

Immediately after the OF test, animals underwent a single 15 min exposure to the FST. Two-way ANOVA revealed significant effects of strain ($F_{1,94}$=32.674, ***p*<0.001**), but not of treatment ($F_{5,94}$=1.394, p>0.05) or strain x treatment interaction ($F_{5,94}$=1.237, p>0.05), on time spent immobile, a measure of behavioural despair (Figure 4.6A). Further *post hoc* test failed to reveal a significant difference in duration of immobility in vehicle-treated WKY versus SD rats. High dose of U50488 increased duration of immobility in the WKY rats compared to vehicle controls (WKY-U50488 5 mg/kg vs WKY-Vehicle, ***p*<0.05**); this effect was not observed in the SD counterparts. The data were further analysed as temporal profile divided into 5 min time bins (Table 4.3). RM ANOVA revealed significant effects of time ($F_{1.828,151.719}$=90.548, ***p*<0.001**) and time x strain interaction ($F_{1.828,151.719}$=43.460, ***p*<0.001**) in tests of within-subjects effects and a significant overall effect of strain ($F_{1,83}$=32.666, ***p*<0.001**) in tests of between-subjects effects on duration of immobility. However, further *post hoc* test at each time bin indicated that there was no significant difference between relevant groups.

Active behaviours, swimming and climbing, were also rated and analysed for the 15 min trial period. Two-way ANOVA revealed significant effect of strain ($F_{1,94}$=33.501, ***p*<0.001**), but not of treatment ($F_{5,94}$=1.953, p>0.05) or strain x treatment interaction ($F_{5,94}$=1.437, p>0.05), on time spent swimming but *post hoc* analysis did not yield any significant difference between relevant groups (Figure 4.6B). Two-way ANOVA revealed significant effects of strain ($F_{1,94}$=4.449, ***p*<0.05**) and treatment ($F_{5,94}$=6.026, ***p*<0.001**), but not of strain x treatment interaction ($F_{5,94}$=0.312, p>0.05), on time spent climbing (Figure 4.6C). However, further *post hoc* tests failed to show any difference between relevant groups. Further analysis of the swimming and climbing data in 5 min time bins did not reveal any significant effect of the factors in RM ANOVA.

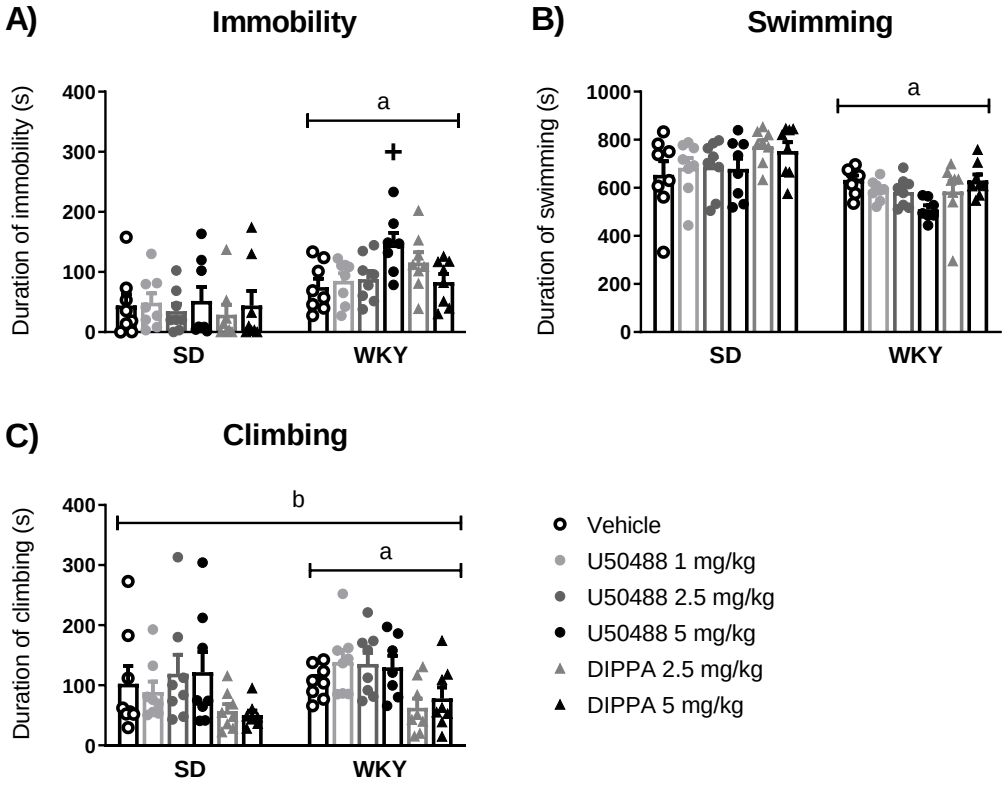


Figure 4.6: Effects of U50488 (1, 2.5, and 5 mg/kg, s.c.) and DIPPA (2.5 and 5 mg/kg, s.c.) on duration of (A) immobility, (B) swimming, and (C) climbing over 15 min in the FST in SD and WKY rats (Experiment 1). Data are expressed as mean ± SEM, n = 7-8/group. a: effect of strain (p<0.05), b: effect of treatment (p<0.001), ^{+}p<0.05 (WKY-U50488 5 mg/kg vs WKY-Vehicle). FST: forced swim test, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Table 4.3: Temporal profile of the effects of U50488 (1, 2.5, and 5 mg/kg, s.c.) and DIPPA (2.5 and 5 mg/kg, s.c.) on duration of immobility in the FST in SD and WKY rats (Experiment 1). Data are presented in 5 min time bins and expressed as mean ± SEM, n = 7-8/group. FST: forced swim test, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Groups	Time spent (s)		
	0-5 min	5-10 min	10-15 min
SD-Vehicle	12.07±6.73	13.86±8.40	18.13±6.66
SD-U50488 1 mg/kg	10.00±3.44	16.09±7.30	22.86±7.96
SD-U50488 2.5 mg/kg	5.04±2.43	9.12±4.27	20.69±6.20
SD-U50488 5 mg/kg	15.75±7.14	17.83±8.57	18.35±10.73
SD-DIPPA 2.5 mg/kg	2.83±1.80	13.60±8.26	12.50±8.46
SD-DIPPA 5 mg/kg	12.41±7.11	18.00±9.34	13.72±8.04
WKY-Vehicle	5.26±2.53	27.77±6.22	41.64±7.30
WKY-U50488 1 mg/kg	9.78±2.75	34.79±6.07	40.65±6.36
WKY-U50488 2.5 mg/kg	3.45±1.28	35.95±7.25	48.76±5.95
WKY-U50488 5 mg/kg	18.25±4.40	55.42±9.44	72.05±10.73
WKY-DIPPA 2.5 mg/kg	9.12±4.29	45.84±8.16	60.63±10.21
WKY-DIPPA 5 mg/kg	5.20±1.54	27.90±7.28	50.16±8.18

4.3.2. Experiment 2

4.3.2.1. Effects of U50488 and DIPPA, alone or in combination, on formalin-evoked nociceptive and general exploratory behaviours in WKY and SD rats

After a week of washout and reallocation of rats to new treatment groups according to a Latin square design, Experiment 2 was carried out to evaluate the effects of systemic administration of U50488 (1 and 2.5 mg/kg) and DIPPA (2.5 mg/kg), alone or in

combination, in the F-CPA paradigm. On conditioning day 2, rats received an intraplantar formalin injection into the right hindpaw and were observed for 60 min. Formalin-evoked nociceptive behaviour (assumed to primarily reflect the sensory component of the noxious stimulus) was analysed temporally in 5 min bins (Figure 4.7). Two-way RM ANOVA revealed significant effects of time ($F_{4.680,383.743}$=38.975, ***p*<0.001**), time x strain ($F_{4.680,383.743}$=14.791, ***p*<0.001**), time x treatment ($F_{23.399,383.743}$=1.955, ***p*<0.01**), and time x strain x treatment interaction ($F_{23.399,383.743}$=1.784, ***p*<0.05**) in tests of within-subjects effects on formalin-evoked nociceptive behaviour. Two-way RM ANOVA also found significant overall effects of strain ($F_{1,82}$=239.482, ***p*<0.001**), treatment ($F_{5,82}$=6.371, ***p*<0.001**), and strain x treatment interaction ($F_{5,82}$=3.594, ***p*<0.01**) in tests of between-subjects effects on formalin-evoked nociceptive behaviour. Further *post hoc* analysis showed that the vehicle-treated WKY rats exhibited higher formalin-evoked nociceptive behaviour in the first 20 min post-formalin injection, compared to SD counterparts [T_{0-20} (in 5 min bins): WKY-Vehicle vs SD-Vehicle, ***p*<0.05**; Figure 4.7A], confirming their hyperalgesic phenotype to noxious inflammatory stimulus (Burke et al., 2010; Rea et al., 2014).

Figure 4.7B and 4.7C illustrate the effects of subcutaneous administration of U50488 and/or DIPPA on nociceptive responding induced by formalin in SD and WKY rats, respectively. U50488 (1 and 2.5 mg/kg) significantly reduced formalin-evoked nociceptive behaviour in a dose-related manner in the SD rats, compared to vehicle-treated controls [T_{5-10}, T_{20-50} (in 5 min bins): SD-U50488 1 mg/kg vs SD-Vehicle, ***p*<0.05**; T_{5-10}, T_{20-60} (in 5 min bins): SD-U50488 2.5 mg/kg vs SD-Vehicle, ***p*<0.05**]; this effect was not seen in the WKY counterparts. Pretreatment with DIPPA failed to attenuate U50488-induced antinociceptive effect in the SD rats [T_{0-60} (in 5 min bins): SD-U50488 1 mg/kg+DIPPA 2.5 mg/kg vs SD-U50488 1 mg/kg, p>0.05; T_{0-60} (in 5 min bins): SD-U50488 2.5 mg/kg+DIPPA 2.5 mg/kg vs SD-U50488 2.5 mg/kg, p>0.05]. In addition, DIPPA (2.5 mg/kg), administered alone, significantly reduced formalin-evoked nociceptive behaviour in the SD rats [T_{5-10}, T_{35-60} (in 5 min bins): SD-DIPPA 2.5 mg/kg vs SD-Vehicle, ***p*<0.05**], but not in the WKY counterparts.

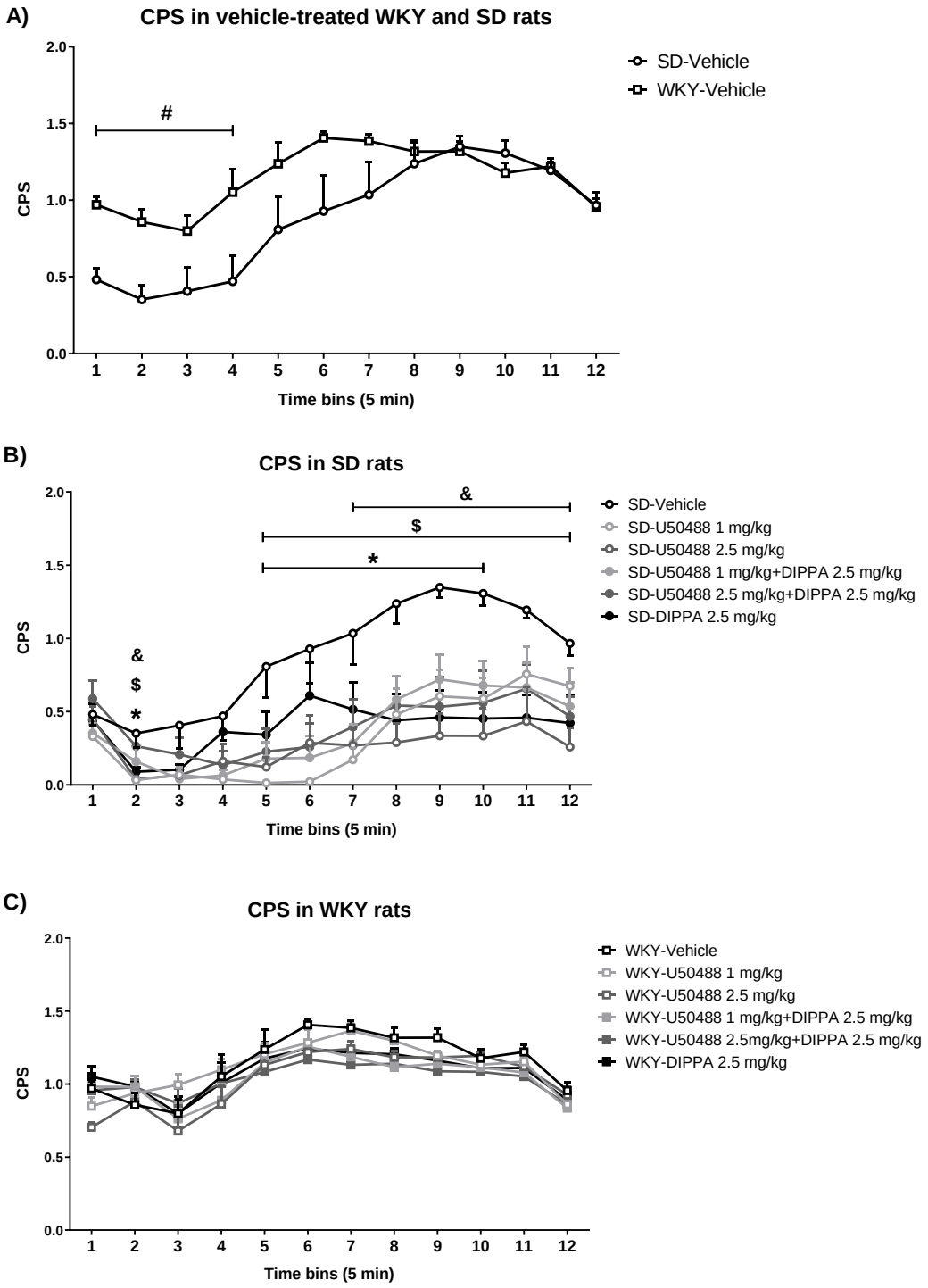

A)
CPS in vehicle-treated WKY and SD rats
SD-Vehicle
WKY-Vehicle
#
CPS
Time bins (5 min)
B)
CPS in SD rats
SD-Vehicle
SD-U50488 1 mg/kg
SD-U50488 2.5 mg/kg
SD-U50488 1 mg/kg+DIPPA 2.5 mg/kg
SD-U50488 2.5 mg/kg+DIPPA 2.5 mg/kg
SD-DIPPA 2.5 mg/kg
&
$
*
CPS
Time bins (5 min)
C)
CPS in WKY rats
WKY-Vehicle
WKY-U50488 1 mg/kg
WKY-U50488 2.5 mg/kg
WKY-U50488 1 mg/kg+DIPPA 2.5 mg/kg
WKY-U50488 2.5mg/kg+DIPPA 2.5 mg/kg
WKY-DIPPA 2.5 mg/kg
CPS
Time bins (5 min)

Figure 4.7: (A) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving vehicle injection systemically. Temporal profile of the effects of U50488 (1 and 2.5 mg/kg, s.c.) and DIPPA (2.5 mg/kg, s.c.), alone or in combination, on formalin-evoked nociceptive behaviour in (B) SD and (C) WKY rats (Experiment 2). Data are presented in 5 min time bins and expressed as mean ± SEM, n = 7-8/group. *$p<0.05$ (SD-U50488 1 mg/kg or SD-U50488 1 mg/kg+DIPPA 2.5 mg/kg vs SD-Vehicle), $^{\$}p<0.05$ (SD-U50488 2.5 mg/kg or SD-U50488 2.5 mg/kg+DIPPA 2.5 mg/kg vs SD-Vehicle), $^{\&}p<0.05$ (SD-DIPPA 2.5 mg/kg vs SD-Vehicle), $^{\#}p<0.05$ (WKY-Vehicle vs SD-Vehicle). CPS: composite pain score, s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

General locomotor activity and defaecation were also analysed during the 60 min formalin trial on conditioning day 2 (Table 4.4). A significant main effect ($\chi^2(11)=31.600$, ***p*<0.01**) was observed on distance moved in the formalin-paired compartment in KW test. *Post hoc* analysis showed that the vehicle-treated WKY rats exhibited a significant reduction in distance moved compared to SD controls (WKY-Vehicle vs SD-Vehicle, ***p*<0.001**). The high dose (2.5 mg/kg) of U50488, alone or in combination with DIPPA, significantly reduced distance moved in the SD rats (SD-U50488 2.5 mg/kg or SD-U50488 2.5 mg/kg+DIPPA 2.5 mg/kg vs SD-Vehicle, ***p*<0.01**) but not in the WKY counterparts. Significant main effects were revealed on rearing ($\chi^2(11)=44.109$, ***p*<0.001**), grooming ($\chi^2(11)=50.526$, ***p*<0.001**), and faecal pellets excreted ($\chi^2(11)=30.006$, ***p*<0.01**) during formalin trial but further *post hoc* tests indicated there were no significant differences between relevant groups. In addition, formalin-induced hindpaw oedema was analysed (Figure 4.8). Two-way ANOVA revealed a significant effect of strain ($F_{1,93}=5.867$, ***p*<0.05**), but not of treatment ($F_{5,93}=0.147$, $p>0.05$) or strain x treatment interaction ($F_{5,93}=0.621$, $p>0.05$), on paw oedema. Further *post hoc* analysis showed there were no significant differences between relevant groups.

Table 4.4: Effects of U50488 (1 and 2.5 mg/kg, s.c.) and DIPPA (2.5 mg/kg, s.c.), alone or in combination, on general exploratory behaviours (distance moved, rearing, and grooming) and defaecation (number of pellets produced) during the 60 min formalin trial on conditioning day 2 (Experiment 2). Data are expressed as mean ± SEM except for defaecation which is expressed as median with IQR, n = 7-8/group. $^{**}p<0.01$ (SD-U50488 2.5 mg/kg or SD-U50488 2.5 mg/kg+DIPPA 2.5 mg/kg vs SD-Vehicle), $^{\#\#}p<0.01$ (WKY vs SD counterpart). s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Groups	Distance moved (cm)	Rearing (s)	Grooming (s)	Defaecation
SD-Vehicle	7127.08± 594.08	58.78±17.13	29.08±8.65	2(4)
SD-U50488 1 mg/kg	3931.13± 409.75	30.06±5.66	5.56±2.43	2.50(2.50)
SD-U50488 2.5 mg/kg	**2792.63± 267.71****	54.34±26.80	3.45±2.72	2.50(4.50)
SD-U50488 1 mg/kg + DIPPA 2.5 mg/kg	3935.94± 525.34	49.46±13.02	13.30±4.82	0.50(3.75)
SD-U50488 2.5 mg/kg + DIPPA 2.5 mg/kg	**3037.82± 415.89****	25.13±6.35	7.32±3.15	2(3)
SD-DIPPA 2.5 mg/kg	4981.58± 881.45	55.84±18.16	17.86±4.77	1(3)
WKY-Vehicle	**3785.91± 314.62$^{\#\#}$**	14.10±4.33	57.71±9.81	0(0)
WKY-U50488 1 mg/kg	2940.45± 227.51	9.68±6.20	24.26±4.95	0(0)
WKY-U50488 2.5 mg/kg	3418.55± 365.04	6.79±1.15	19.55±5.93	4(3.75)
WKY-U50488 1 mg/kg + DIPPA 2.5 mg/kg	2578.19± 202.31	10.77±3.74	46.12±11.06	0(0.75)
WKY-U50488 2.5 mg/kg + DIPPA 2.5 mg/kg	2793.12± 260.66	9.43±3.88	30.83±7.79	0(0.75)
WKY-DIPPA 2.5 mg/kg	2913.90± 306.91	6.79±3.34	45.01±4.99	0(0)

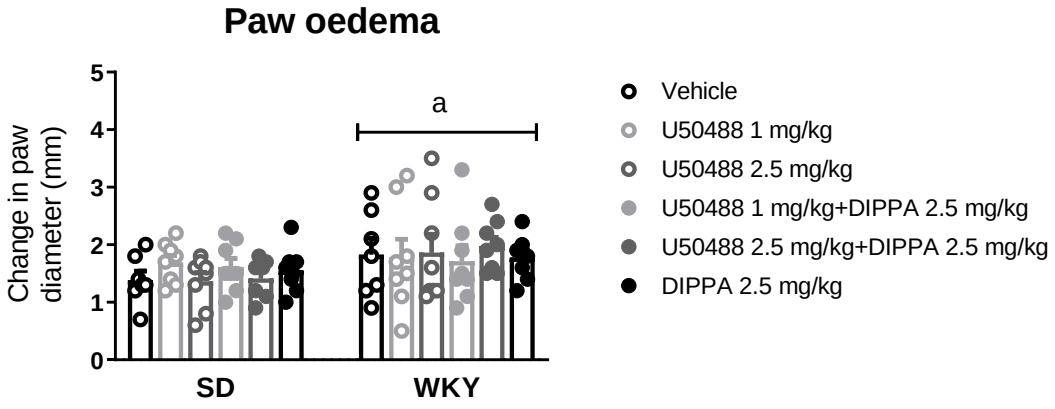


Figure 4.8: Effects of U50488 (1 and 2.5 mg/kg, s.c.) and DIPPA (2.5 mg/kg, s.c.), alone or in combination, on formalin-induced hind paw oedema in SD and WKY rats (Experiment 2). Paw oedema was measured as the difference in diameter (mm) of the right hindpaw immediately before intraplantar formalin injection on conditioning day 2 and after euthanasia on postconditioning day. Data are expressed as mean ± SEM, n = 7-8/group. a: effect of strain (p<0.05). s.c.: subcutaneous, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

4.3.2.2. Effects of U50488 and DIPPA, alone or in combination, on formalin-induced conditioned place aversion in WKY and SD rats

The affective component of the noxious (inflammatory) stimulus was assessed in the F-CPA paradigm. Locomotor activity of the animals on the preconditioning day was recorded and analysed. Two-way ANOVA revealed a significant effect of strain ($F_{1,78}$=17.261, ***p*<0.001**), but not of treatment ($F_{5,78}$=0.276, p>0.05) or strain x treatment interaction ($F_{5,78}$=0.640, p>0.05), on the distance moved in the arena. *Post hoc* analysis did not yield any significant difference between relevant groups (Figure 4.9A). Furthermore, time spent in each compartment on the preconditioning day was recorded to evaluate any initial preference in the animals for one of the conditioning compartments. Any rat spending more than 900 s (75% of total trial time) in one compartment was excluded from further analysis of CPA (Tanimoto et al., 2003; Lu et al., 2011). Since baseline locomotor activity in a novel environment differs between SD and WKY rats

(Paré, 1989c, 1994; Tejani-Butt et al., 1994), preference was analysed in the two strains separately. Two-way ANOVA revealed significant effects of side and side x treatment interaction, but not of treatment, on time spent in the compartments in both SD (side: $F_{1,85}$=16.605, ***p*<0.001**; treatment: $F_{5,85}$=0.001, p>0.05; side x treatment: $F_{5,85}$=3.039, ***p*<0.05**) and WKY (side: $F_{1,71}$=47.561, ***p*<0.001**; treatment: $F_{5,71}$=0.016, p>0.05; side x treatment: $F_{5,85}$=3.910, ***p*<0.01**) rats. Further *post hoc* analysis revealed a significant initial preference for the vertical stripe/cinnamon-paired compartment over horizontal stripe/acetic acid-paired compartment in the animals of the following groups: SD-U50488 2.5 mg/kg+DIPPA 2.5 mg/kg, WKY-U50488 1 mg/kg, and WKY-U50488 2.5 mg/kg (Figure 4.9B and 4.9C).

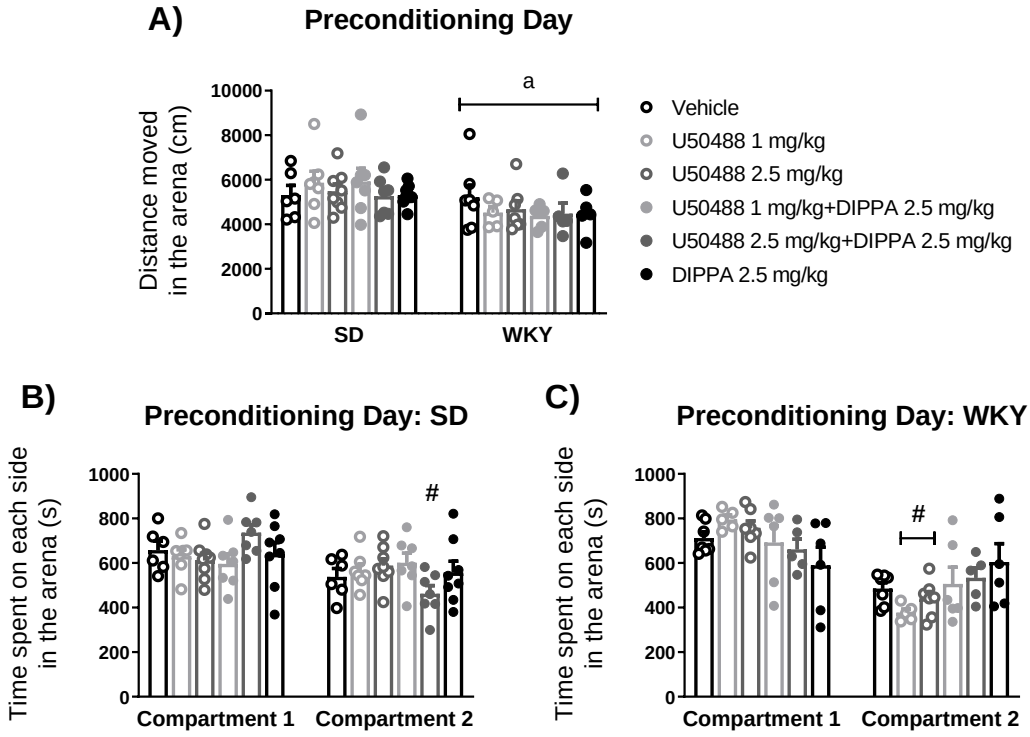


Figure 4.9: (A) Distance moved and (B and C) time spent in each conditioning compartment on the preconditioning day in SD and WKY rats. Compartment 1 had vertical black and white stripes on the walls and a cinnamon scent associated with it while compartment 2 had horizontal black and white stripes with odour of 1% acetic acid. Data are expressed as mean ± SEM, n = 5-8/group. a: effect of strain (p<0.001), $^{\#}p$<0.05 (compartment 2 vs compartment 1). SD: Sprague-Dawley, WKY: Wistar-Kyoto.

As on the preconditioning day, locomotor activity and time spent in each compartment were recorded on the postconditioning day. Two-way ANOVA revealed a significant effect of strain ($F_{1,78}$=34.493, ***p*<0.001**), but not of treatment ($F_{5,78}$=1.055, p>0.05) or strain x treatment interaction ($F_{5,78}$=0.217, p>0.05), on distance moved in the arena during the 20 min trial period. *Post hoc* analysis indicated there was no significant difference between relevant groups (Figure 4.10A). Two-way ANOVA revealed a significant effect of treatment ($F_{5,78}$=2.418, ***p*<0.05**), but not of strain ($F_{1,78}$=0.186, p>0.05) or strain x treatment interaction ($F_{5,78}$=0.953, p>0.05), on CPA score (Figure 4.10B). The vehicle-treated SD rats did not spend less time in the formalin-paired compartment on the postconditioning day relative to preconditioning day (as shown by a positive CPA score), indicating F-CPA did not occur. In both SD and WKY rats, U50488 tended to enhance place aversion induced by formalin; this effect was sensitive to pretreatment with DIPPA. However, further interpretation of the effects of drugs was hindered since the control group (SD-vehicle rats) in our experiment failed to exhibit formalin-induced place aversion.

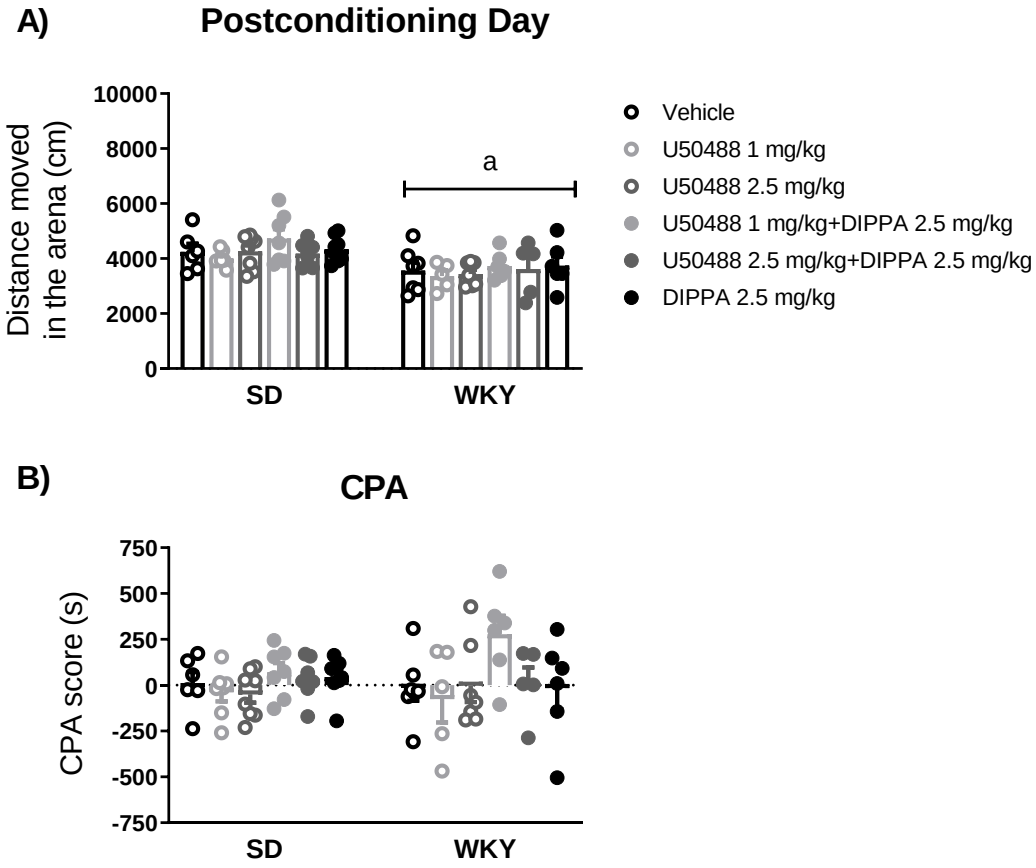


Figure 4.10: (A) Distance moved in the arena on the postconditioning day in SD and WKY rats. (B) Effects of U50488 (1 and 2.5 mg/kg, s.c.) and DIPPA (2.5 mg/kg, s.c.), alone or in combination, on CPA score in SD and WKY rats. CPA score was calculated as the difference in time spent in the formalin-paired compartment between the pre- and postconditioning sessions, Data are expressed as mean ± SEM, n = 5-8/group. a: effect of strain (p<0.001). CPA: conditioned place aversion, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

4.3.3. Expression of genes encoding KOP and PDYN in brain regions associated with pain and affect in naïve WKY and SD rats

The expression of the genes encoding KOP and PDYN in discrete brain regions involved in pain and negative affect was assessed in naïve WKY and SD rats using RT-qPCR. The brain tissues used here were the same ones described in Chapter 2 that were obtained from the behaviourally naïve cohort of rats and used for the analysis of expression of the genes encoding MOP and POMC. Analysis of gene expression revealed that WKY rats had lower level of KOP mRNA in the PFC than SD rats ($t_{8.757}$=2.368, ***p*<0.05**; Figure 4.11A). A trend was observed for lower level of KOP mRNA in the PAG in WKY versus SD rats. In addition, WKY rats showed higher levels of PDYN mRNA in the PFC ($t_{10.791}$=-3.573, ***p*<0.01**) and hippocampus ($t_{12.717}$=-4.455, ***p*<0.01**), compared to SD counterparts (Figure 4.11B). Moreover, a strong trend was found for lower expression of the gene encoding PDYN in the PAG in WKY rats, compared to SD counterparts. There was no difference in the expression of the genes encoding KOP and PDYN in the other regions analysed between the two strains.

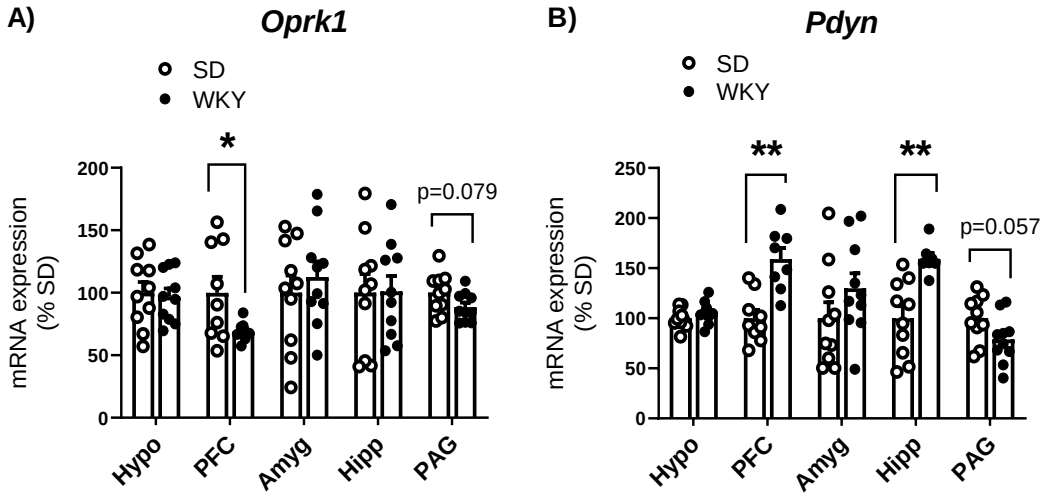


Figure 4.11: The expression of the genes encoding KOP and PDYN in discrete brain regions involved in pain and negative affect in naïve WKY and SD rats. Data are expressed as mean ± SEM, n = 7-10/group. **p*<0.05, ***p*<0.01 (vs SD). Amyg: amygdala, Hypo: hypothalamus, Hipp: hippocampus, KOP: kappa opioid receptor, PAG: periaqueductal grey, PFC: prefrontal cortex, PDYN: prodynorphin, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

4.4. Discussion

The results presented in this chapter demonstrate that systemic administration of U50488 (the prototypic KOP agonist) was antinociceptive in the SD and WKY rats in the hot plate test. In comparison, the WKY rats were hyporesponsive to the antinociceptive effects of U50488 in the formalin test, compared to SD counterparts. Interestingly, DIPPA, when administered systemically on its own, had antinociceptive effects in the SD, but not in the WKY, rats upon exposure to noxious heat or inflammatory stimuli. We attempted to investigate the effects of modulating KOP on the aversive responding to a noxious inflammatory (formalin) stimulus in a place conditioning test. However, the F-CPA paradigm requires further refinement in our laboratory condition. In addition, we did not obtain further evidence for anxiolytic or antidepressant-like effects of KOP antagonism in either strain. Finally, WKY rats exhibited differential expression of the genes encoding KOP and PDYN in cortical and limbic brain regions, compared to SD counterparts.

The experience of pain encompasses both sensory and affective dimensions. Numerous pharmacological and genetic studies have shown that activation of the KOP produces antinociception (Millan et al., 1987; Piercey and Einspahr, 1989; Bianchi and Panerai, 1993; Terner et al., 2005; Gallantine and Meert, 2008; Bagdas et al., 2016) but also induces dysphoria or aversion in rodents (Pfeiffer et al., 1986; Shippenberg and Herz, 1987; Bals-Kubik et al., 1993; Land et al., 2008; Tejeda et al., 2013). Our study is the first to investigate the effects of modulating KOP on nociceptive (sensory) and aversive (affective) responding to noxious stimulation in the WKY rat strain, a genetic model of altered nociceptive responding co-occurring with mood associated behavioural deficits. With respect to nociceptive responding to the acute heat stimulus, U50488 produced antinociception in both SD and WKY rats, albeit there is some difference in the minimum effective dose between the two strains. However, this strain difference of U50488 potency could be due to the modest increment in response latency observed in the vehicle-treated WKY rats at the 30 min time point, resulting in a lack of statistically significant antinociceptive effect of U50488 in the WKY rats at that time point. As mentioned earlier, the WKY rats are hypersensitive to stress (Pardon et al., 2002) and, therefore, it is possible that due to repeated exposure to the hot plate within short intervals may affect their nocifensive behaviour. Although not statistically significant, there seems to be a biologically significant effect of U50488 (2.5 and 5 mg/kg) in the WKY rats at the 30 min time point as the majority of rats in these treatment groups reached maximum

response latency. It is worth considering here whether the 40 s cut-off used in the protocol might be acting as a "ceiling effect" and hence affecting the behavioural outcome. For a chosen temperature in the hot plate test, cut-off times are carefully designed and strictly adhered to (Bannon and Malmberg, 2007). A lower cut-off time would make detection of drug effects/response difficult while a higher cut-off may induce unnecessary nociceptive stimulation and tissue damage. The 40 s cut-off is a well-established limit in the literature for the temperature range used in the hot plate test in the present study (Deuis et al., 2017). Thus, an alternative could be to reduce the stimulus intensity (i.e. using lower hot plate temperature) and subsequently increase the cut-off time to minimise any possible effect of time limit on behavioural responses.

Next, in the formalin trial of Experiment 2, systemic administration of U50488 (1 and 2.5 mg/kg) significantly reduced formalin-evoked nociceptive behaviour in the SD rats but no such effect was observed in the WKY counterparts. These novel findings clearly indicate that the WKY rats exhibit reduced sensitivity to the antinociceptive effects of U50488 in the formalin model of persistent inflammatory pain, compared with SD rats. One interesting point to note here is that this strain difference in sensitivity to KOP agonist also seems to be specific to pain modality (thermal vs chemical/inflammatory). Previous studies have reported that pain modality could influence potency/efficacy of opioids in both humans (Koltzenburg et al., 2006; Kindler et al., 2011) and rodents (Hayes et al., 1987; Morgan et al., 2006). The two noxious stimuli, heat and chemical, used herein activate different subsets of transducer proteins in the nociceptive primary afferents. Our results suggest that there could be a difference in the KOP-induced modulation of these afferents involved in detecting heat and/or chemical in WKY versus SD rats, such that U50488 fails to attenuate formalin-evoked nociception in WKY rats but is effective in reducing heat nociception in both strains.

The WKY rats also exhibited reduced distance moved during the formalin trial, compared to SD rats, indicating their hallmark hypolocomotor trait as shown previously (Tejani-Butt et al., 1994; Burke et al., 2010). The doses of U50488 used in the F-CPA component (Experiment 2) of the study were chosen based on the results of the earlier dose response experiment (Experiment 1) with the intention to avoid any potential effect of U50488 on locomotor activity. In Experiment 1, an indirect effect on locomotor activity of the high doses (2.5 and 5 mg/kg) of U50488 was observed in the SD rats (increased closed arm duration) in the EPM, but no such effect of U50488 (1-5 mg/kg) was found in the OF trial

in either strain. However, the 60-min formalin trial (Experiment 2) revealed a locomotor-impairing effect of U50488 when administered at dose 2.5 mg/kg (alone or in combination with DIPPA) in SD rats. Previous studies have reported that U50488 at doses ≥3 mg/kg reduces exploratory activity in rodents (Ukai and Kameyama, 1985; Jackson and Cooper, 1988; Simonin et al., 1998), possibly through KOP-mediated inhibition of the midbrain dopaminergic system (Di Chiara and Imperato, 1988; Kunihara et al., 1993; Kuzmin et al., 2000). Of note, this locomotor reducing effect of U50488 was not evident in the WKY rats, further highlighting a strain-related difference in behavioural responses to U50488. Studies in KOP-deficient mice confirm that KOP is central in mediating pharmacological effects of U50488 *in vivo* (Simonin et al., 1998). Thus, it seems plausible to suggest that differences in KOP expression and/or functionality may explain differential responsivity to KOP agonism in WKY versus SD rats, and that changes in the KOP system may underpin some of the characteristic behaviours exhibited by the WKY rat strain.

Another finding in our study is that DIPPA produced antinociceptive effects in the SD rats upon exposure to both noxious acute heat and tonic persistent inflammatory stimuli. Structure-affinity relationship studies indicate that DIPPA has high affinity and selectivity for KOP over MOP and DOP and exhibits irreversible KOP binding (Chang et al., 1994b; Schwartz et al., 1997). A series of experiments conducted by Chang and coworkers reveal paradoxical KOP agonist- and antagonist-like effects of DIPPA that also vary across species (Chang et al., 1994b, 1994a). For example, DIPPA reduced [^{3}H]U69593 (a selective KOP agonist) binding by 87% in guinea pig brain membranes but inhibited twitch response (agonist-like activity) in guinea pig ileum and mouse vas deferens preparations *in vitro*. Moreover, DIPPA was antinociceptive for up to 4 h in the mouse abdominal stretch assay that was sensitive to pretreatment with nor-BNI (the prototypic KOP antagonist). Conversely, DIPPA antagonised U50488-induced antinociception (up to 48-72 h) in the mouse tail flick test. The authors suggested that DIPPA has transient KOP-agonist effects (≤4 h) followed by long-lasting KOP-antagonist effects (up to 48-72 h) *in vivo* in mice. These findings have prompted subsequent studies to administer the drug 24 or 48 h before behavioural testing (Kuzmin et al., 2000; Terner et al., 2005; Carr and Lucki, 2010; Carr et al., 2010). Nevertheless, systemic administration of DIPPA yielded mixed results. DIPPA exhibits KOP agonist-like activity in a pigeon drug discriminatory procedure and mild antinociceptive effects in rat

model of acute pain (Terner et al., 2005), suggesting that it has some agonist efficacy at KOP. Others have reported either attenuation (Kuzmin et al., 2000; Terner et al., 2005) or no effect (Burton and Gebhart, 1998) of DIPPA on U50488-induced behavioural responses in rodents. Taken together, these mixed agonist/antagonist results suggest that DIPPA may function as a low efficacy or partial agonist (Terner et al., 2005), which may partly explain the antinociceptive effects of DIPPA seen in our study. However, DIPPA also attenuates preclinical measures of anxiety- and depression-like behaviours in several rodent models (Carr and Lucki, 2010; Carr et al., 2010) that is in agreement with its KOP antagonist profile. Recent studies from the Porreca group suggest that blocking the KOP may alleviate ongoing pain (Xie et al., 2017; Navratilova et al., 2019). The long-acting KOP antagonist nor-BNI, specifically in the amygdala, prevents stress-induced hyperalgesia in a rat model of migraine (Xie et al., 2017). These effects were replicated with a selective short-acting KOP antagonist (Xie et al., 2017), further highlighting clinical potential of KOP antagonists as a novel class of therapeutics in persistent and chronic pain. Hence, another possibility is that DIPPA acts as an antagonist *in vivo* at the time point of behavioural testing in the present study and mediates antinociceptive effects perhaps via the amygdalar KOP circuit. Further *in vitro* as well as *in vivo* characterisation of DIPPA is warranted to fully elucidate its behavioural profile at the KOP. It should also be noted that the WKY rats exhibited hyporesponsivity to DIPPA-induced antinociceptive effects in the formalin test but not in the hot plate test. This further supports the notion that a possible deficit in the KOP system, at least in the components of descending pain pathway that modulate the sensory dimension of a noxious stimulus, may underlie the WKY hyperalgesic phenotype to persistent inflammatory stimuli.

The neural systems responsible for the emotional-affective component of pain have gained much attention in recent times. As mentioned earlier, the KOP play a key role in driving the affective dimension of pain (Cahill et al., 2014; Liu et al., 2019b). Therefore, we compared the effects of systemic administration of U50488 and DIPPA on the affective component of noxious persistent inflammatory pain in WKY versus SD rats using the F-CPA paradigm. However, the control group (vehicle-treated SD rats) in our experiment failed to exhibit avoidance behaviour from the distinct environment in the place conditioning apparatus that was paired to noxious formalin injection. This outcome also limits our scope in interpreting any potential drug effects in the two strains. There are a few important avenues in the F-CPA protocol used in this study that can be modified

in order to make the behavioural paradigm work in our laboratory conditions. Firstly, irrespective of the strain, there was a clear natural bias in the animals for the compartment paired with cinnamon scent over the other compartment with acetic acid odour as shown on preconditioning day. The odour stimuli can be changed to other scents such as strawberry and peppermint in order to reduce this initial compartment bias. Moreover, the visual cue to distinguish the two conditioning compartments can be enhanced by using more distinct patterns such as dots and stripes. Next, we used an unbiased approach in our design which, when pairing a given compartment with the noxious stimulus (formalin), does not consider any innate preference of the animal for one of the conditioning compartments upon initial exposure. In this respect, some laboratories have additional pre-test days in the protocol before preconditioning day to assess any initial preference of the animals for a conditioning compartment. In order to nullify any initial preference of an animal, another useful approach is to use the bias design: pairing formalin treatment with the preferred compartment from preconditioning day (Tzschentke, 1998). Appropriate control needs to be included such as counterbalancing the formalin-pairing with preferred and non-preferred compartment within a treatment group. The DIPPA injection immediately after exposure to the compartment on conditioning day 1 (non-formalin day) may have some aversive aspect due to the acute stress associated with drug injection, which probably can be avoided by habituating the rats to the procedure of injection (e.g. by administering vehicle) before conditioning days. Thus, further pilot studies with the above mentioned refinements are necessary to validate the F-CPA paradigm under our laboratory conditions.

The present study also examined the effects of systemic administration of U50488 and DIPPA on anxiety- and depression-related behaviours in the WKY and SD rats. We employed a battery of behavioural tests (hot plate, EPM, OF, and FST) in Experiment 1, which was similar to the experimental design used in the study for investigating the effects of morphine in the two strains in Chapter 2. In contrast with previous reports, the vehicle-treated WKY rats in this study did not display reduced open arm and centre zone activities in the EPM and OF tests, respectively, compared to SD counterparts (Gentsch et al., 1987; Paré, 1992; Tejani-Butt et al., 1994; Malkesman and Weller, 2009; Burke et al., 2010, 2016). This discrepancy is possibly due to the methodological differences between our study and those published, as mentioned earlier in Chapter 2. Acute stress due to prior exposure to hot plate tests may induce freezing in the WKY rats (Paré, 1989c;

Zafar et al., 1997; Nosek et al., 2008), affecting their behaviours in subsequent tests. In addition, when exposed to novel aversive (e.g. bright light) environment, WKY rats display increased immobility (Paré, 1989c; Berton et al., 1997; O'Malley et al., 2010), which was evident in our study in the measure of time spent in the centre of the OF. In addition, the vehicle-treated WKY rats displayed marked hypolocomotor activity in the OF compared to SD controls, in agreement with previous reports (Paré, 1994; McAuley et al., 2009; Burke et al., 2010, 2016). When exposed to the 15-min FST, the vehicle-treated WKY rats exhibited progressive (numerical) increase in the duration of immobility (a measure of behavioural despair) over the course of the trial, compared to SD counterparts.

Systemic administration of U50488 did not affect anxiety-related behaviours in the EPM and OF tests in either strain. Although activation of KOP induces anxiogenic effects (Narita et al., 2006a; Vunck et al., 2011; Smith et al., 2012; Valdez and Harshberger, 2012; Gillett et al., 2013), some studies have also reported anxiolytic effects of U50488 (Privette and Terrian, 1995; Kudryavtseva et al., 2005) in rodents. Discrepancies among these published studies may be due to, but are not limited to, differences in experimental models used, doses employed, light and test conditions, and animal species/strains used. Moreover, since the KOP are widely expressed in the brain, specific regions may have opposite and compensatory roles in the regulation of anxiety by KOP (Wang et al., 2016). Therefore, further studies are needed to understand effects of KOP agonists on anxiety-like behaviours. In the FST, high dose of U50488 increased immobility in WKY, but not in SD, rats, suggesting an enhanced prodepressive-like effect induced by activating the KOP (Mague et al., 2003; Carlezon et al., 2006) in the WKY rats. A significant effect of DIPPA on anxiety- and depression-related behaviours in either strain at the doses tested in our study was not observed. Past reports have demonstrated anxiolytic and antidepressant-like effects of DIPPA in the WKY rats, compared with SD counterparts (Carr and Lucki, 2010; Carr et al., 2010). Methodological differences (such as different anxiety tests and higher dose (10 mg/kg) of DIPPA in the FST) between these studies and ours may account for the observed discrepancy.

We next explored whether a difference in the basal expression of the genes encoding KOP and PDYN in discrete brain regions (implicated in processing pain and affect/emotion) in WKY and SD rats account for the differences in the phenotype between the two strains. Our data showed a region-dependent difference in the expression of these genes: lower

level of KOP mRNA in the PFC and higher levels of PDYN mRNA in the PFC and hippocampus in behaviourally naïve WKY rats, compared to SD counterparts. Trends for lower levels of KOP and PDYN mRNA were observed in the PAG in WKY versus SD rats. A recent study by Burke and colleagues reported higher expression of KOP mRNA in the amygdala but a trend for lower expression of KOP mRNA in the PFC in WKY versus SD rats (Burke et al., 2019). The authors did not find any strain-related difference in the expression of PDYN mRNA in the PFC and hippocampus. Methodological differences (e.g. singly- vs pair-housed naïve rats) between these two studies may account for the discrepancy here. Reduced mRNA expression of KOP in key pain processing regions (e.g. in the PFC and PAG) may result in hyporesponsive effects of KOP modulators in the WKY rats. Elevated dynorphin mRNA expression in the corticolimbic regions (e.g. in the PFC and hippocampus) may contribute to the stress hypersensitivity displayed by the WKY rats (Shirayama et al., 2004). However, differences in gene expression may not translate to differences in the protein level or function of the receptor/peptide. Thus, further investigations are needed to determine whether the endogenous peptide level and expression/functionality of the receptor protein are also altered.

In conclusion, the differential KOP-induced behavioural responses obtained in this study in the WKY versus SD rats suggest that a maladaptive KOP system in the WKY rats may underlie their hyperalgesic phenotype, particularly to a noxious inflammatory stimulus. However, DIPPA did not produce strain-specific changes to anxiety- and depression-related behaviours, possibly due to the low dose employed in the present study. Nevertheless, further investigation is warranted to fully characterise the pharmacological profile of DIPPA at the KOP. Region-dependent differential expression of KOP and dynorphin may underpin some of the behavioural deficits in the WKY rat model.

5. Chapter 5: Comparison of pain-, anxiety-, and cognition-related behaviours in the complete Freund's adjuvant model of chronic inflammatory pain between Wistar-Kyoto and Sprague-Dawley rats

5.1. Introduction

According to The International Association for the Study of Pain (IASP), chronic pain is defined as pain that persists or recurs for more than 3 months and is associated with both emotional distress and functional disability including interference with activities of daily life and participation in social roles (Treede et al., 2019). It is a major unmet clinical problem in current times, having a significant impact on the individual and imposing a huge socioeconomic burden. Epidemiological studies have indicated that chronic pain associated with persistent inflammation is one of the leading causes of global non-fatal health loss in terms of years lost to disability (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). Despite being a highly prevalent health problem worldwide, the current treatment strategies against chronic inflammatory pain are limited, perhaps owing to poor understanding of the exact underlying neurobiological mechanisms (Borsook et al., 2014). In addition, persistent inflammatory pain states may trigger a cascade of changes in psychological processes (including perception, attention, mood, motivation, learning, and memory) along with the sensory symptoms, allodynia (pain in response to normally non-painful stimulus) and hyperalgesia (increased pain response to painful stimuli) (Simons et al., 2014a). Moreover, affective disorders (anxiety and depression) (Asmundson and Katz, 2009; Scott et al., 2016; Stubbs et al., 2016) and cognitive dysfunction (Moriarty et al., 2011; Shin et al., 2012) are highly comorbid with chronic pain, further contributing to the debilitating nature of the disorder and hence posing a significant challenge with regards to treatment strategy.

Injecting a noxious chemical irritant such as formalin, complete Freund's adjuvant (CFA), or carrageenan into the paws or joints of rodents can be used to mimic the key features of chronic inflammatory pain in humans (Burma et al., 2017). Of these, the CFA-induced inflammation (Stein et al., 1988) lasts usually for 1-3 weeks, allowing for long-term studies of localised inflammation-related persistent pain responses (Zhang and Ren,

2011). Injection of CFA into the joints or cutaneous/subcutaneous tissues results in mechanical allodynia (Stein et al., 1988; Chaplan et al., 1994; Soignier et al., 2011) and thermal (heat and cold) hyperalgesia (Hargreaves et al., 1988; Moriarty et al., 2012) in rodents. In addition to affecting sensory modality, CFA induces the emotional-affective component of pain. Intraplantar hindpaw CFA injection produces place aversion in rodents (Zhang et al., 2011; Liu et al., 2019b) that is blocked by KOP antagonist JDTic without affecting tactile allodynia (Liu et al., 2019b), suggesting KOP may modulate the ongoing tonic-aversive component of chronic inflammatory pain. In the place escape/avoidance paradigm, which dissociates sensory and affective dimensions of pain experience, CFA-injected rodents avoid the preferred location when the injured paw was stimulated, reflecting the aversive nature of the noxious stimulation (Labuda and Fuchs, 2000; Boyce-Rustay et al., 2010; Refsgaard et al., 2016). Escape/avoidance behaviour was attenuated in rats by injecting an astroglial toxin directly into the anterior cingulate cortex (ACC) (Chen et al., 2012), an important brain region in the integration of mood and nociception (Johansen et al., 2001; LaGraize et al., 2004). This result suggests that the activation of astrocytes in the ACC may contribute to the affective component of pain. Studies have also reported enhanced anxiety- (Narita et al., 2006a; Chen et al., 2013; do Nascimento and Leite-Panissi, 2014; Refsgaard et al., 2016) and depression-like (Shi et al., 2010; Kim et al., 2012; Grégoire et al., 2014) behaviours in several rodent models of chronic inflammatory pain including the CFA model. Moreover, CFA-induced inflammatory pain in rodents have been associated with impairments in multiple cognitive domains including spatial learning, recognition memory, attention, and social interaction (Cain et al., 1997; Lindner et al., 1999; Pais-Vieira et al., 2009; Parent et al., 2012; Grégoire et al., 2014; Yang et al., 2014; Amaral et al., 2015). However, no study to date has investigated the influence of genetic background on the interaction of pain and anxiety and/or cognition in the CFA-induced chronic inflammatory pain model.

The inbred WKY rat is a prominent and frequently used rodent model of anxiety and depression. Compared to other rat strains, WKY rats exhibit increased immobility in the FST (Paré and Redei, 1993; Tejani-Butt et al., 1994; Rittenhouse et al., 2002; Burke et al., 2010), enhanced anxiety-like behaviour (Gentsch et al., 1987; Paré, 1994; Burke et al., 2010, 2016) and social avoidance (Paré, 2000; Pardon et al., 2002; Nam et al., 2014), diminished activity when exposed to a novel environment (Paré, 1989c; Pardon et al., 2002), and impaired cognition (Gonzales et al., 2015). Moreover, WKY rats show

augmented neuroendocrine stress responses (Redei et al., 1994; Rittenhouse et al., 2002). In addition, WKY rats exhibit increased sensitivity to visceral (Gunter et al., 2002; Gibney et al., 2010; O' Mahony et al., 2013), thermal (heat) (Burke et al., 2010), and inflammatory (formalin) (Burke et al., 2010; Rea et al., 2014) noxious stimuli. Furthermore, WKY rats display exacerbated mechanical allodynia/hyperalgesia and enhanced depression-like behaviour in two rat models of chronic pain (peripheral nerve injury and CFA-induced temporomandibular joint inflammation), compared to Wistar counterparts (Zeng et al., 2008; Wang et al., 2012), suggesting an influence of genotype on exacerbated nociceptive responding and negative affect in chronic pain. We hypothesised that the effects of CFA-induced chronic inflammation on behavioural domains related to nociceptive responding, negative affect, and cognition will be exaggerated in the WKY rat strain, compared to SD counterparts. The specific aims of the study conducted herein are as follows:

- To characterise nociceptive responding to mechanical and thermal (radiant heat) stimuli prior to and after inducing chronic inflammation using CFA in WKY versus SD rats.
- To assess the emotional-affective component of pain following CFA-induced chronic inflammation in SD and WKY rats.
- To compare anxiety-like behaviour and general locomotor activity following CFA-induced chronic inflammation in SD and WKY rats.
- To assess effects of CFA-induced chronic inflammatory pain on social, recognition, and spatial memory in SD and WKY rats.

5.2. Materials and methods

5.2.1. Animals

Experiments were carried out in male WKY and SD rats (7-8 weeks old, 180-230 g on arrival; Envigo, Bicester, UK). Rats were maintained under a standard 12:12 h light/dark cycle (lights on from 07:00-19:00 h) in a temperature- (21±2°C) and humidity-controlled (45-55%) room throughout the study. Upon arrival, all animals were housed in groups of 3-4 per cage in plastic bottom cages (45x20x20 cm^3) containing 3Rs basic bedding (>99% recycled paper; 3Rs Lab, Fibrecycle Ltd, North Lincolnshire, UK) and sizzle nest material (LBS Biotechnology, Horley, UK). Following 5 days of acclimatisation to the unit, animals were housed singly in cages for the rest of the study. An additional cohort of male SD and WKY rats (n=16/strain) were used in this study as conspecifics for the three-chamber sociability test. The conspecific rats were pair-housed according to the strain for the duration of the study. Food (14% protein rodent diet; Envigo, Bicester, UK) and water were available *ad libitum*. All experimental procedures were carried out during the light phase between 08:00 and 18:00 h. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway. The work was carried out under license from the Health Products Regulatory Authority in the Republic of Ireland and in accordance with EU Directive 2010/63. The study was also designed and carried out in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

5.2.2. Complete Freund's adjuvant injection

Immunogenic complete Freund's adjuvant (CFA; Sigma-Aldrich, Wicklow, Ireland), consisting of heat-killed and dried *Mycobacterium tuberculosis* in an 85% paraffin oil and 15% mannide monooleate suspension, was used to induce a chronic inflammatory pain state (Stein et al., 1988). Rats were injected once with 100 µL CFA (1 mg/mL) in the plantar surface of the right hindpaw under brief isoflurane anaesthesia (3% in 0.8 L/min O_2). Control rats received an intraplantar needle insertion into the right hindpaw under the same conditions. The duration of anaesthesia in all cases was under 2 min. After a brief recovery period, animals were returned to their home cages.

5.2.3. Study design

The experimental timeline is illustrated in Figure 5.1. Baseline nociceptive responses to cutaneous mechanical and thermal (heat) stimulations were assessed in the von Frey and Hargreaves' tests, respectively, for each rat. Two sets of baseline testing were conducted based on previous work in our group showing reduction in response thresholds/latencies at the second baseline testing session relative to the initial session. Baseline paw withdrawal thresholds in the von Frey test were determined 5 and 2 days before CFA injection. Similarly, baseline paw withdrawal latencies in the Hargreaves' test were measured 4 and 1 days prior to CFA injection. After baseline testing, animals were randomly assigned to a treatment condition (control or CFA injection), resulting in 4 experimental groups: SD-control, SD-CFA, WKY-control, and WKY-CFA (Table 5.1). Following CFA/control injections (Day 0), animals underwent a series of behavioural investigations: (1) mechanical allodynia was assessed using von Frey test on Days 1, 6, and 22; (2) thermal hyperalgesia was determined using Hargreaves' test on Days 2, 7, and 23; (3) place escape/avoidance paradigm evaluated the affective component of pain on Day 11; (4) anxiety-like behaviours were assessed on Day 13 using elevated plus maze and open field tests; and (5) different cognitive behaviours were evaluated using the three-chamber sociability test (Day 15), novel object recognition test (Day 18), and T-maze spontaneous alternation test (Day 20-21). On Day 24, rats were euthanised by decapitation.

Table 5.1: Summary of experimental groups. The animals received i.pl. needle insertion (control) or 100 µL CFA injection into their right hindpaw under brief isoflurane anaesthesia. CFA: complete Freund's adjuvant, i.pl.: intraplantar, n: number, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Group	Strain	Treatment (i.pl., right hindpaw)	n
1	SD	Control	10
2	SD	CFA	10
3	WKY	Control	10
4	WKY	CFA	10

Figure 5.1: Schematic of the study outlining the timeline of behavioural tests. 3-CST: three-chamber sociability test, CFA: complete Freund's adjuvant, EPM: elevated plus maze, HG: Hargreaves' test, inj: injection, NOR: novel object recognition test, OF: open field, PEAP: place escape/avoidance paradigm, TM: T-maze test, VF: von Frey test.

5.2.4. Behavioural testing

5.2.4.1. Sensory testing

5.2.4.1.1. von Frey test for mechanical allodynia

The von Frey (VF) test was used to assess mechanical allodynia (sensitivity to a non-noxious stimulus). The apparatus (Figure 5.2A) consisted of a six-chambered arena made of clear Perspex front and back and white, melamine-coated chipboard lateral walls. The dimensions (14x20x25 cm^3) of each chamber were such that the rat could move freely. A Perspex lid with air-holes was positioned on top of the arena during habituation and testing periods to prevent animals escaping from the arena. The arena was placed on a raised wire-mesh flooring so that the experimenter could access the hindpaws of the rats from below. 6 rats were tested in each session. The arena was cleaned thoroughly between the sessions using a warm soapy solution.

On the test day, rats were habituated to the arena for 15 min. The test was carried out using the up-and-down method described by Dixon (Dixon, 1980), where the rats received a maximum of 9 nylon VF filament stimulations (Touch Test Sensory Evaluator #58011, Stoelting, IL, USA), starting with the 2 g filament. Each filament was applied once, perpendicular to the plantar surface of the hindpaw, and targeting the area (Figure 5.2B) at the base of the third and fourth digits (from medial to lateral) according to previous protocol used by the group. A sufficient uniform force was applied to cause slight buckling of the filament for approximately 5 s or until a positive response was observed. Flinching, licking, or a brisk withdrawal of the paw upon application of the filament or immediately after removal of the filament was considered a positive response. During the test, both hindpaws were stimulated with VF filaments (alternating between paws, starting with the contralateral paw). The responses to the 2 g filament for the contralateral paws of all 6 rats were first measured; the responses to the 2 g filament for the ipsilateral paws were collected next. If a positive response was observed using the 2 g filament, filaments of lower weights (down) were applied in descending order until no positive responses were observed in that paw. If no response was observed using the 2g filament, filaments of higher weights (up) were applied in ascending order until a positive response was observed in that paw. After the first change in response pattern in either case, additional applications were performed: when no response, the next filament with a higher force was tested, and when a positive response, the next lower force filament was

tested (up-and-down). In all cases, a maximum of 9 stimulations was applied to each paw. The response pattern generate a code that is associated to a constant, *k*, in the table by Dixon (Dixon, 1980). The mechanical withdrawal threshold (g) for each paw was calculated using the formula: $10^{[\log(\text{last filament})+k*0.3]}$

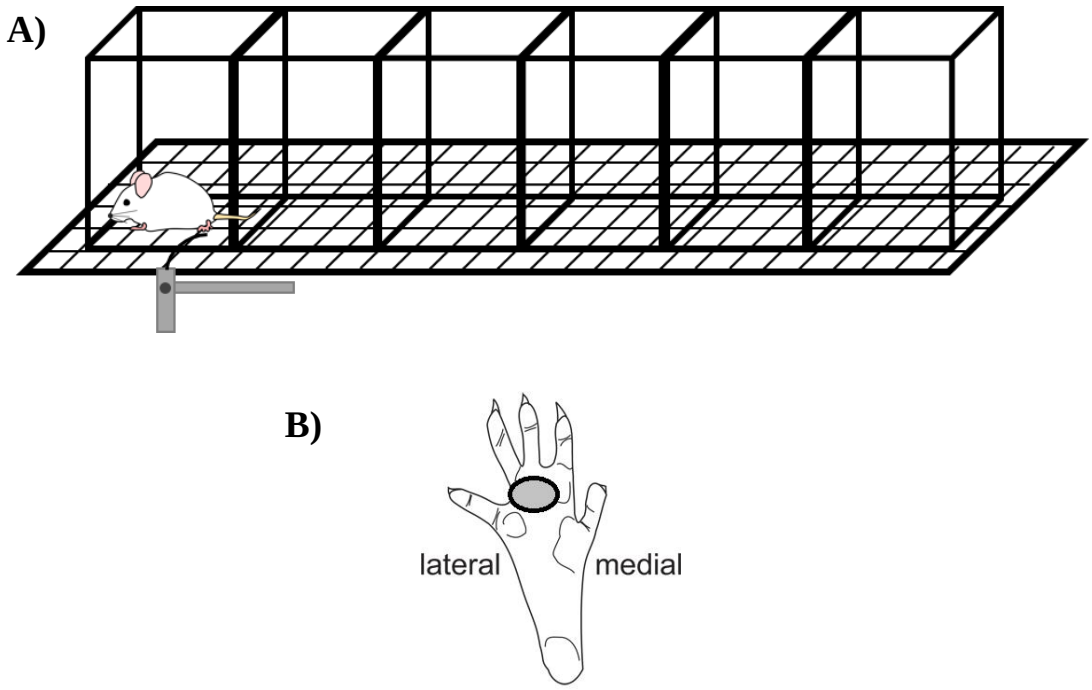


Figure 5.2: von Frey testing for mechanical allodynia. (A) Schematic diagram of the apparatus used. (B) Plantar surface of the right hindpaw of a rat, showing the area (shaded) at which the von Frey filament was targeted.

5.2.4.1.2. Hargreaves' test for thermal hyperalgesia

Thermal (heat) hyperalgesia (increased sensitivity to a noxious stimulus) was assessed in the Hargreaves' (HG) test (Hargreaves et al., 1988) using a commercially available apparatus (Plantar Analgesia Meter, IITC Life Sci Inc, CA, USA). The apparatus (Figure 5.3) consisted of six clear Perspex chambers (11x20x15 cm^3 per chamber) and placed on top of a raised glass panel that was heated to 30±1°C. A Perspex lid with air-holes was placed on top of the arena during habituation and testing periods to prevent animals escaping from the arena. Rats were placed in individual chambers and allowed to habituate for 15 min. A moveable radiant light heat source was positioned underneath the glass plate. Using a guide light (idle intensity of 1%) and an adjustable angled mirror, a

focused beam of radiant light (active intensity of 30% corresponding to 53°C) was applied from below to the plantar surface of the hindpaw, targeting the area between the third and fourth digits (from medial to lateral), same location as for the VF filaments. The latency to flinch, lick or withdraw the hindpaw was recorded. Once the animal responded, the pushbutton was pressed to simultaneously stop the timer and deactivate the heat source, which then returned to idle intensity. A cut-off point of 20 s was set to avoid any tissue damage. If the animal did not respond positively within the cut-off time, a latency of 20 s was recorded. 6 animals were tested in each session. The withdrawal latencies of the ipsilateral paws for all 6 animals were measured first, then the latencies of the contralateral paws were collected. In this way, the ipsi- and contralateral paws of each animal in a given session were tested four times (alternating between the paws). The average withdrawal latency (s) for each paw over the four trials was calculated. The arena was cleaned thoroughly between the sessions using a warm soapy solution.

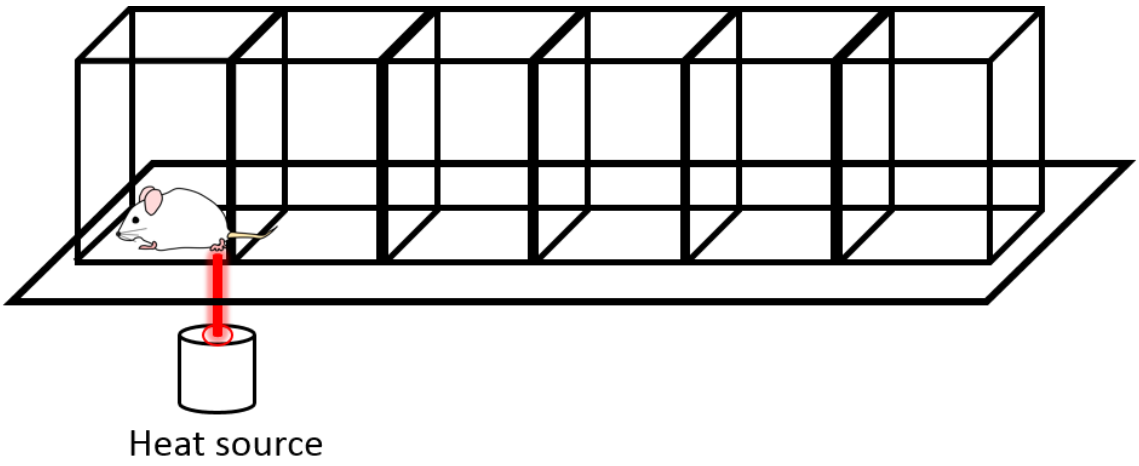


Figure 5.3: Schematic diagram of the apparatus used for Hargreaves' testing to assess thermal (heat) hyperalgesia.

5.2.4.2. Place escape/avoidance paradigm

The place escape/avoidance paradigm (PEAP) measures and dissociates the affective/motivational and sensory components of pain processing (Fuchs and McNabb, 2012). In the current study, we conducted PEAP on Day 11 post-CFA injection as described previously (Labuda and Fuchs, 2000). The apparatus (Figure 5.4) consisted of a clear Perspex arena (30x30x30 cm^3) positioned on top of a wire mesh. The arena was divided into two compartments by a central partition with a small opening, allowing the animal to move freely between the two sides. One compartment was transparent and defined as the "light side" (20-22 lux). The other compartment was called the "dark side" (0-1 lux); it was covered with black paper and had a wooden black lid on top to minimise entry of light. A video camera was located on the top of the light side to record the behaviours during the test session. The arena was cleaned thoroughly between animals with a warm soapy solution.

The animals were always placed into the light side of the arena facing away from the central opening/dark side. One day prior to testing (Day 10 post-CFA injection), animals were initially habituated for 30 min to the test apparatus. On the test day (Day 11 post-CFA injection), rats were given an additional 10 min to habituate to the arena. Testing began immediately thereafter with the animal receiving a noxious mechanical stimulation using a suprathreshold VF filament (60 g) to the plantar surface of the hindpaws (same location as for the VF test) at an interval of 15 s for 30 min. During this 30-min behavioural testing, the rat was allowed unrestricted movement throughout the arena. The ipsilateral (CFA injected) paw was stimulated when the animal was in the dark side and the contralateral paw was stimulated when it was in the light side of the arena. This sets up a situation in which the animal has a choice to move to the aversive light (non-preferred) side in order to escape and/or avoid noxious mechanical stimulation to the injured area in the dark (normally preferred) side. The animal was considered to be in a given side of the arena where both its hindpaws were located. The behavioural responses to noxious stimuli (criteria for a positive response was similar to those used for VF testing) were recorded manually during the trial. The behaviours of the animals (time spent and number of entries into the light side) in the arena were recorded and analysed later using the EthoVision software (EthoVision XT, version 11.5; Noldus, Wageningen, Netherlands). An increase in the time spent/entries in the light side of the arena reflects the degree of aversion to noxious stimulation.

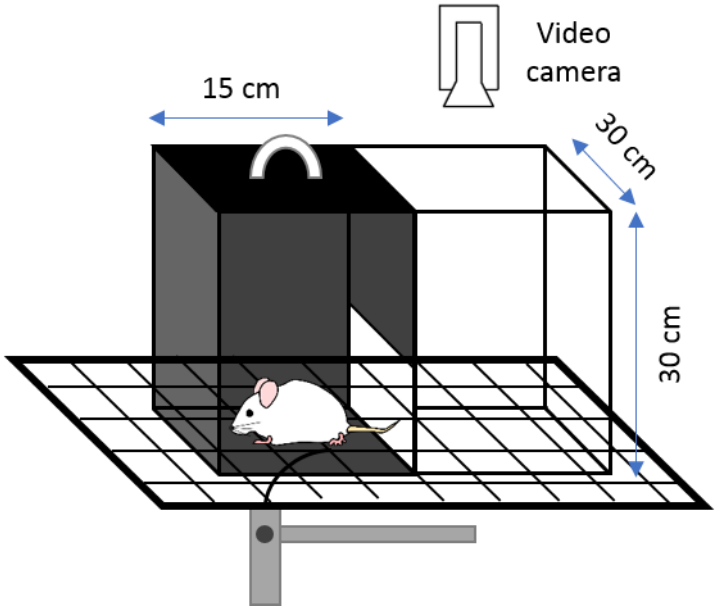


Figure 5.4: Schematic diagram of the apparatus used for place escape/avoidance paradigm. The animal was stimulated with 60 g von Frey filament on the ipsilateral paw when on the dark side and on the contralateral paw when on the light side of the arena.

5.2.4.3. Tests of anxiety-related behaviour

5.2.4.3.1. Elevated plus maze test

On Day 13 post-CFA injection, the animals were assessed for anxiety-like behaviours first in the 5-min elevated plus maze (EPM) test. The test was performed described previously in Chapter 2 (see section 2.2.4.2.1). The arena was cleaned between animals using a warm soapy solution. Using EthoVision software, the following behaviours were quantified: time spent and number of entries in the open arms, closed arms, and centre square.

5.2.4.3.2. Open field test

Following EPM test, rats were immediately exposed to the open field (OF) for 5 min. The test was carried out as described previously in Chapter 2 (see section 2.2.4.2.2). The exception was that the floor of the circular arena used in this study was made up of reflective aluminium sheet. The arena was cleaned between animals using a warm soapy solution. EthoVision system was used to track distance moved in the arena, time spent

and number of entries in the centre zone, and latency to leave the centre zone. Number of pellets excreted during the trial was also counted.

5.2.4.4. Tests of cognition

5.2.4.4.1. Three-chamber sociability test

The three-chamber sociability test (3-CST) was used to assess social recognition behaviour and social memory (Crawley, 2004) on Day 15 post-CFA injection. The rectangular arena (90.5x46x40 cm^3) was made up of opaque grey plastic sheets, except for the front panel that was of transparent Plexiglass. It consisted of three compartments: outer left, middle, and outer right, each separated with a transparent Plexiglass partition, such that the animal could see the other compartments though the partition. The outer left and right compartments mirror each other. The light intensity levels in each compartment (46x30.16 cm^2) were between 30-40 lux. Each partition contained a small hole giving the animal free access in and out of the compartment. In each of the outer compartments, a wire mesh insert could be fitted 10 cm from the back wall of the compartment to create a restricted area into which a conspecific rat was placed during testing. In this way, the test rat could interact with the novel conspecific animal by pure olfactory, visual and auditory cues.

The test consisted of 3 consecutive sessions (Figure 5.5): habituation, sociability, and social novelty/preference trials, each lasting for 10 min. In the habituation phase, the test animal was placed to the centre of the middle compartment and allowed to freely explore the empty arena for 10 min. The test rat was then confined to the middle compartment by blocking the holes in the partition walls (using bottles filled with water). The wire mesh inserts were placed into both outer left and right compartments. A novel conspecific rat (C1) was then confined behind the wire mesh insert in one of the outer compartments. A lid was placed onto each restricted cage to stop the conspecific from escaping. The placement of the first conspecific rat into the outer left or right compartment was randomly allocated for each trial to avoid a side preference and counterbalanced within a treatment group. Also, the conspecifics used for each trial were of the same strain as the test rat. The water bottles were then removed, and the test rat was allowed to freely explore and interact with the conspecific rat or empty cage insert for another 10 min (sociability trial). The test rat was again confined to the middle compartment in a similar manner and a second novel conspecific rat (C2) was placed behind the wire mesh insert

of the other outer compartment. The test rat was then allowed to freely explore and interact with the first conspecific (now familiar, C1) or the second conspecific (novel, C2) rat for a further 10 min (social novelty/preference trial). After this, the test rat was removed from the arena and returned to its home cage. The conspecific rats were also removed and returned to their home cage. The arena was cleaned thoroughly between animals using warm soapy water and dried before starting the next trial.

A video camera located on top of the arena was used to record behaviours during testing sessions that were later analysed using the EthoVision system. Distance moved (cm) by the test animal in the arena in each session was tracked using EthoVision. Social interactions were manually scored as times spent engaging in investigatory behaviours including sniffing, rearing/climbing against the wire mesh, and active interaction with conspecific animal. In addition, two indices were calculated to evaluate social approach and memory, respectively, in the 3-CST (Baronio et al., 2015): (1) sociability index, defined as time spent interacting with the conspecific animal (C1) relative to the time spent interacting with the empty cage, and (2) social novelty/preference index, defined as time spent interacting with the familiar conspecific animal (C1) relative to the time spent interacting with the novel conspecific animal (C2). The equations used to calculate these indices are given below (Table 5.2):

Table 5.2: Equations for calculating the indices for the assessment of behaviours in the 3-CST. T_{C1}: time spent interacting with first conspecific animal, T_{cage}: time spent interacting with empty cage, T_{C2}: time spent interacting with second conspecific animal.

Index	Trial	Equation
Sociability index	Sociability	$(T_{C1} - T_{cage})/(T_{C1} + T_{cage})$
Social novel/preference index	Social novel/preference	$(T_{C1} - T_{C2})/(T_{C1} + T_{C2})$

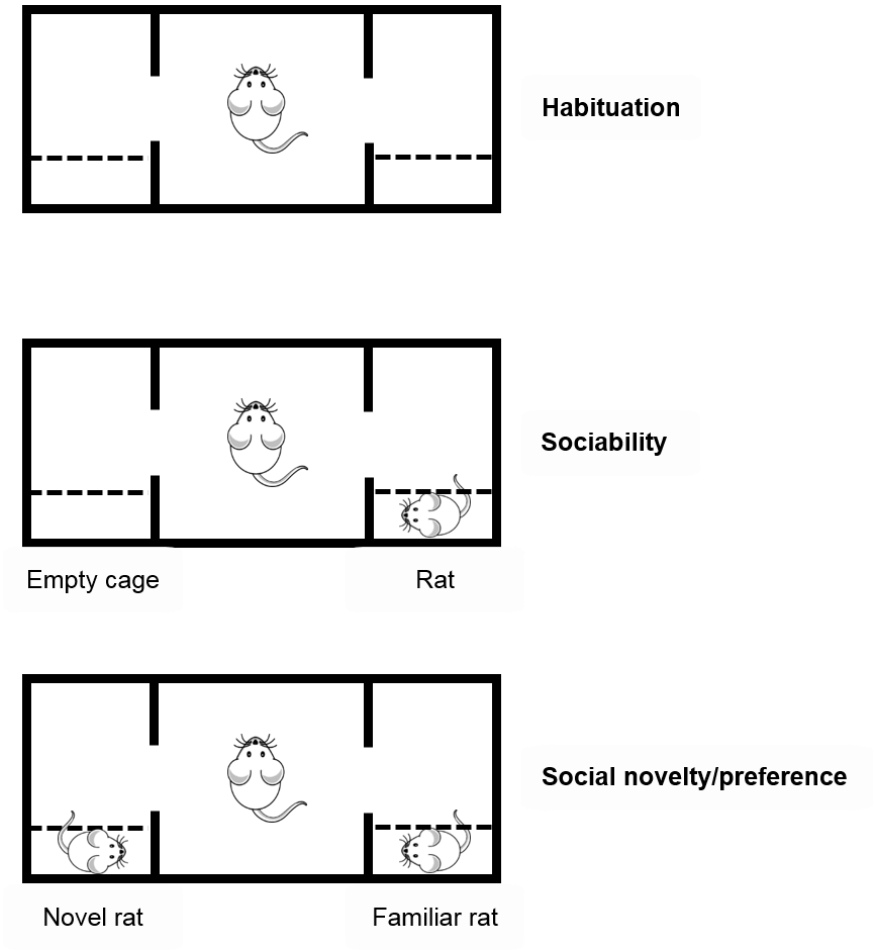


Figure 5.5: Schematic diagram for the three-chamber sociability test apparatus and testing timeline. The test consisted of three consecutive 10-min sessions: habituation trial (exploration of the empty arena), sociability trial (exploration of a novel conspecific rat verses an empty cage), and social novelty/preference trial (exploration of the now familiar conspecific rat verses a second novel conspecific rat).

5.2.4.4.2. Novel object recognition test

The novel object recognition (NOR) test primarily relies on the natural tendency of the animal to spend more time exploring new object rather than a previously encountered one and was first described as a measure of recognition memory by Ennaceur and Delacour (Ennaceur and Delacour, 1988). The test was performed according to protocols described previously (Fahey et al., 2008; Callaghan et al., 2018) with slight modifications. Testing was conducted in the same circular arena (diameter: 70 cm) used previously for OF test

(Figure 5.6A). The arena was illuminated at a constant light intensity of 100±10 lux at the floor level. A video camera positioned 35 cm above the floor of the arena was used to record behaviours during all sessions for subsequent analysis. The objects used during the sessions were 500 mL unlabelled transparent plastic Coca-Cola® bottles (with black plastic caps and filled with water) and an abstract plastic structure (base area: 5x5 cm^2, height: 16 cm) constructed using green, white, and blue toy blocks (Playskool Clipo™ blocks). The base of the objects were secured to the floor of the arena with adhesive so that they were difficult to displace by the animal.

The NOR test consisted of 3 sessions: habituation, familiarisation day, and test day, conducted on consecutive days (Day 16 to 18 post-CFA injection). On the first day, animals were habituated to the arena in the absence of objects for 10 min. On the second (familiarisation) day, three identical objects (Coca-Cola® bottles) were placed in the arena, approximately 20 cm apart and 15 cm from the arena walls. The rat was placed in the centre of the arena facing away from the objects and allowed to freely explore the arena and objects three times for 5 min, with 5 min inter-trial intervals. After the familiarisation phase with identical objects, the animal was returned to its home cage. On the third (test) day, one of the three objects in the arena was replaced with a novel object (abstract plastic structure constructed with toy blocks). The animal was re-exposed to the arena and objects once for 5 min, allowed to explore freely, and then returned to the home cage. Pictorial representation of the task protocol is shown in Figure 5.6B. The arena and objects were cleaned thoroughly between each exposure using 70% ethanol to remove any odour and olfactory cue. The position of the novel object was alternated between rats in order to minimise potential confounding effects related to orientation bias.

EthoVision software was used subsequently to manually score the behaviours of the animals. Object exploration was evaluated by the time spent interacting with the objects that included sniffing the object, rearing against the object or having the head directed towards the object within 2 cm of the object. Distance moved (cm) by the animal in the arena during testing was also tracked using EthoVision. Two indices were also calculated to assess NOR results: (1) preference index, defined as the time spent preferentially exploring the novel object on the test day relative to the time spent exploring the familiar object in the same position on the familiarisation day, and (2) discrimination index, defined as the time spent exploring the novel object in relation to the time spent exploring

the familiar objects on the test day. The equations used to calculate these indices are given below (Table 5.3):

Table 5.3: Equations for calculating the indices for the assessment of behaviours in the NOR test. T_{O1}: time spent exploring object 1, T_{O2}: time spent exploring object 2, T_{O3}: time spent exploring object 3, T_F: time spent exploring the two familiar objects, T_N: time spent exploring the novel object.

Index	Day	Equation
Preference index	Familiarisation day	$[T_{O3} - \{(T_{O1} + T_{O2})/2\}]/$ $[T_{O3} + \{(T_{O1} + T_{O2})/2\}]$
	Test day	$\{T_N - (T_F/2)\}/\{T_N + (T_F/2)\}$
Discrimination index	Test day	$\{T_N - (T_F/2)\}/\{T_N + (T_F/2)\}$

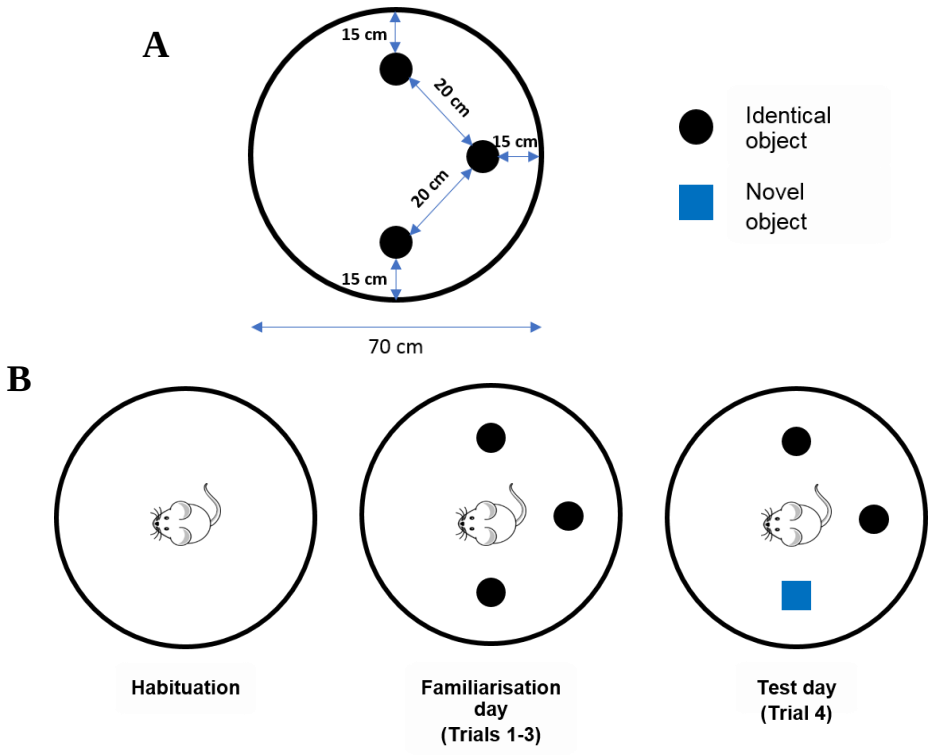


Figure 5.6: Novel object recognition test for testing recognition memory. (A) Schematic diagram for the arena used. (B) Pictorial representation of the test, consisting of 3 sessions on consecutive days: habituation (exploration of the empty arena), familiarisation day (3x5 min trials for exploration of identical objects with 5 min inter-trial interval, and test day (1x5 min trial for exploration of familiar and novel objects).

5.2.4.4.3. T-maze spontaneous alternation test

The T-maze spontaneous alternation test was used to assess spatial memory on Day 20 and 21 post-CFA injection. The task is based on the natural tendency of rodents to explore novel environments, that is, to visit a new arm of the maze rather than a familiar arm upon subsequent exposure. The test was performed according to protocols described previously (Deacon and Rawlins, 2006) with slight modifications. Testing was carried out in a wooden black arena in the shape of a "T" that was elevated 40 cm above the floor of the testing room and enclosed by walls of height 30 cm (see Figure 5.7 for additional dimensions). The guillotine doors and central partition were also made of wood and painted black. The light intensity level at the floor of the maze was between 18-20 lux. A video camera was positioned above the T-maze to record behaviours for subsequent analysis.

The maze was set up for testing with the central partition in place and guillotine doors removed from the entrance of the two goal arms. The animal was placed in the start area and allowed to choose a goal arm (sample phase). Once it chooses a goal arm, the guillotine door was slid down quietly to confine the animal to that arm for 30 s. The central partition was then removed and the animal was returned to the home cage. After raising both the guillotine doors, the rat was returned to the start area of the maze and again allowed to choose any of the two open goal arms (choice phase). The criterion for goal arm selection was that the entire animal (whole body plus the tail tip) be on a goal arm. A cut-off of 90 s was set for selecting a goal arm in both phases of the trial. If the rat failed to select an arm within 90 s, it was removed from the maze and this was considered to be an error of omission. Four rats were tested in the T-maze in one session. Each rat received 10 trials in total (5 trials per day for two days). The mean (1) percentage alternation, (2) error in alternation, and (3) omission of trials were then calculated for each animal. The latency to choose a goal arm in the sample phase was also recorded manually by the experimenter in the room. The maze was cleaned thoroughly between exposures using 70% ethanol to remove any odour or olfactory cue.

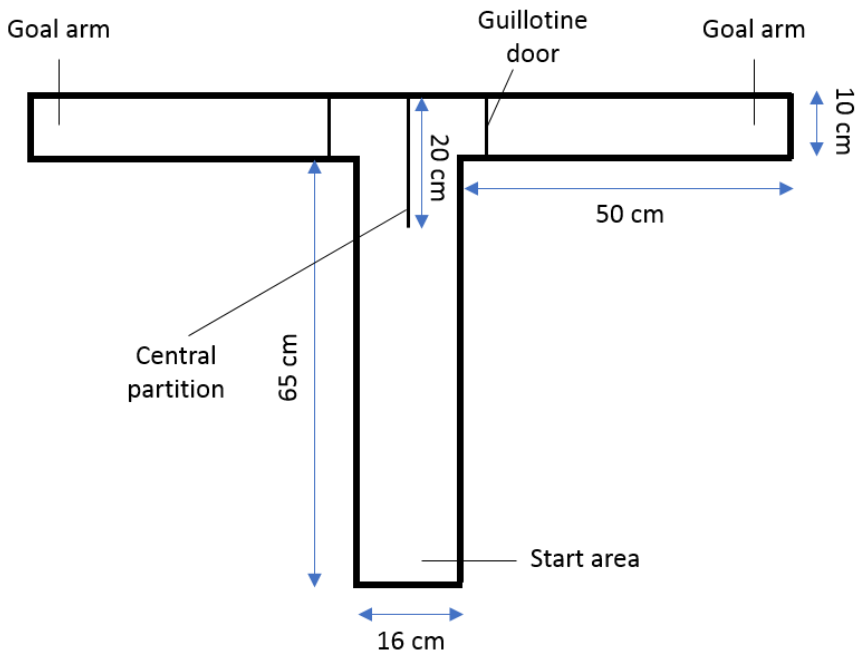


Figure 5.7: Schematic diagram of the apparatus used for testing T-maze spontaneous alternation.

5.2.5. Statistical analysis

The SPSS statistical software (IBM SPSS Statistics, version 24 for Windows; SPSS Inc, Chicago, IL, USA) was used to analyse all data. In all datasets the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was considered an outlier and excluded from subsequent analysis. The normality and homogeneity of variance of the datasets were checked using Shapiro-Wilk and Levene's tests, respectively. The timecourse behavioural data were analysed with repeated measures analysis of variance (RM ANOVA) with strain and CFA injection as between-subject factors and time as within-subject factor. For RM ANOVA, sphericity of the datasets was checked with Mauchly's Test for Sphericity; if the assumption of sphericity was violated, a Greenhouse-Geisser correction was used. Other behavioural data were analysed using the two-way ANOVA with strain and CFA injection as factors. Preference index in NOR test was analysed using three-way ANOVA with day (familiarisation/test) as an additional factor. *Post hoc* Student-Newman-Keuls (SNK) test was carried out for pairwise group comparisons, where appropriate. The level of significance was deemed

$p<0.05$. In addition, analysis of covariance (ANCOVA) was also run with distance moved as covariate for 3-CST and NOR test. If the data were not normally distributed and/or the variance was not homogeneous, three transformations were applied, in the order: square root, natural logarithm, and ranking of the data values to evaluate if parametric statistics can be used. In addition, if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular dataset being analysed, parametric statistical approach was still employed (Moore et al., 2009).

If any dataset was ordinal (e.g. VF test data, defaecation) or found to be not normally distributed and/or the variance was not homogeneous even after transformation, non-parametric statistical analysis was performed using Kruskal-Wallis (KW) one-way analysis of variance by rank, followed by *post hoc* Mann-Whitney U-test (*p*-value was adjusted using Bonferroni-Holm correction for multiple comparisons), where appropriate. Friedman's test followed by Wilcoxon signed-rank test was employed to assess timecourse data of VF test. In all cases statistical analysis of a dataset was carried out for all the groups together. However, in some instances, the groups are graphed separately to present the results clearly in a reader-friendly format. Data are expressed as either mean ± standard error of the mean (S.E.M) or median with interquartile range (IQR) depending on the statistical approach undertaken, parametric or non-parametric, respectively.

5.3. Results

5.3.1. Effects of CFA injection on mechanical allodynia in SD and WKY rats

Friedman's test revealed significant differences in paw withdrawal thresholds among experimental groups for both contralateral ($\chi^2(4)=10.644$, ***p*<0.05**) and ipsilateral ($\chi^2(4)=63.073$, ***p*<0.01**) paws (Figure 5.8). *Post hoc* Wilcoxon test showed that the ipsilateral paw withdrawal threshold at baseline 1 was significantly higher than baseline 2 (***p*<0.05**); no difference in paw withdrawal thresholds was found between baseline 1 and 2 on the contralateral side ($p>0.05$). Hence, subsequent comparisons of paw withdrawal thresholds between pre- and post-CFA time points were performed relative to baseline 2. *Post hoc* Wilcoxon test also revealed that the hindpaw withdrawal threshold on baseline 2 was significantly higher than Days 1, 6, and 22 on the ipsilateral side only in the CFA-injected SD and WKY rats (all three post-CFA time points vs baseline 2, ***p*<0.01**), indicating CFA-induced mechanical allodynia. Wilcoxon test indicated there were no significant differences in contralateral hindpaw withdrawal threshold between baseline 2 and post-CFA time points (all three post-CFA time points vs baseline 2, $p>0.05$).

As shown in Figure 5.8A, significant main effects on paw withdrawal thresholds on Day 1 ($\chi^2(3)=11.936$, ***p*<0.01**), Day 6 ($\chi^2(3)=13.883$, ***p*<0.01**), and Day 22 ($\chi^2(3)=19.816$, ***p*<0.001**) were also observed in KW tests on the contralateral side. Further *post hoc* analysis at each time point indicated that WKY rats, in comparison to SD rats, displayed lower paw withdrawal thresholds on the contralateral side (Day 1: WKY-CFA vs SD-CFA, ***p*<0.01**; Day 6: WKY-control vs SD-control, ***p*<0.01**; WKY-CFA vs SD-CFA, ***p*<0.05**; Day 22: WKY-control vs SD-control, ***p*<0.01**), suggesting increased sensitivity to mechanical stimuli in the WKY rats.

On the ipsilateral side (Figure 5.8B), KW analysis indicated significant main effects on paw withdrawal thresholds at baseline 2 ($\chi^2(3)=9.816$, ***p*<0.05**), Day 1 ($\chi^2(3)=24.447$, ***p*<0.001**), Day 6 ($\chi^2(3)=31.852$, ***p*<0.001**), and Day 22 ($\chi^2(3)=24.453$, ***p*<0.001**). Further *post hoc* tests at each time point revealed that CFA-injected SD and WKY rats exhibited significantly lower paw withdrawal thresholds, compared to respective control counterparts, on Days 1, 6, and 22 post-injection (all post-CFA time points: SD-CFA vs SD-control, ***p*<0.01**; WKY-CFA vs WKY-control, ***p*<0.001**), indicating that CFA induced mechanical allodynia in both strains. Interestingly, CFA-injected WKY rats displayed

greater mechanical allodynia compared with SD counterparts at all three post-CFA time points [WKY-CFA vs SD-CFA: ***p*<0.01** (Day 1), ***p*<0.001** (Day 6), ***p*<0.05** (Day 22)], suggesting enhanced hypersensitivity to evoked mechanical stimulation in CFA-injected WKY rats. WKY rats also exhibited lower ipsilateral paw withdrawal thresholds at discrete time points in the absence of CFA injection, compared to SD rats (baseline 2: WKY-CFA vs SD-CFA, ***p*<0.05**; Day 1: WKY-control vs SD-control, ***p*<0.01**).

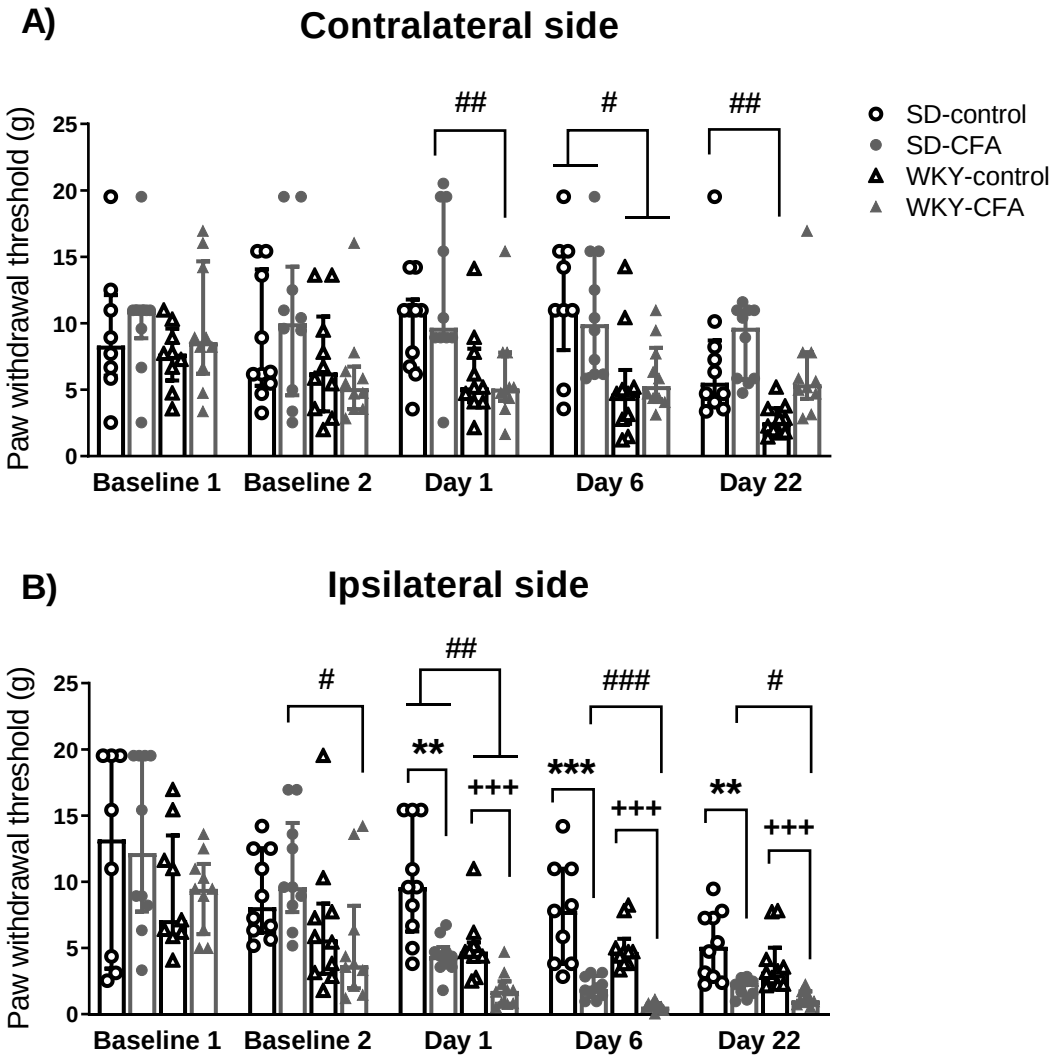


Figure 5.8: Effect of CFA injection on the mechanical withdrawal threshold of the (A) contralateral and (B) ipsilateral hindpaw in SD and WKY rats in the VF test. Data are expressed as median with IQR, n=8-10/group. ***p*<0.01, ****p*<0.001 (SD-CFA vs SD-control), ^{+++}p<0.001 (WKY-CFA vs WKY-control); $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 (WKY vs SD counterpart). CFA: complete Freund's adjuvant, SD: Sprague-Dawley, VF: von Frey, WKY: Wistar-Kyoto.

5.3.2. Effects of CFA injection on thermal (heat) hyperalgesia in SD and WKY rats

RM ANOVA revealed significant effects of time ($F_{4,144}$=63.904, ***p*<0.001**), time x strain ($F_{4,144}$=2.744, ***p*<0.05**), and time x CFA ($F_{4,144}$=45.736, ***p*<0.001**) in tests of within-subjects effects on response latency to noxious heat stimulus on the ipsilateral side. A significant overall effect of CFA ($F_{1,36}$=97.396, ***p*<0.001**) was also observed in tests of between-subjects effects on response latency. *Post hoc* SNK tests at each time point further showed that CFA-injected SD and WKY rats exhibited significantly lower response latency, compared to respective control counterparts, on Days 2, 7, and 23 post-injection (all post-CFA time points: SD-CFA vs SD-control, ***p*<0.05**; WKY-CFA vs WKY-control, ***p*<0.05**), indicating that CFA induced thermal hypersensitivity in both strains (Figure 5.9A). WKY-control rats displayed a transient reduction in ipsilateral hindpaw withdrawal latency, compared to SD-control rats, on Day 2 (WKY-control vs SD-control, ***p*<0.05**).

On the contralateral side (Figure 5.9B), RM ANOVA showed significant effects of time ($F_{3.261,117.392}$=2.619, ***p*<0.05**) and time x strain x CFA interaction ($F_{3.261,117.392}$=2.724, ***p*<0.05**) in tests of within-subjects effects on response latency. However, *post hoc* analysis showed there was no significant difference between relevant experimental groups.

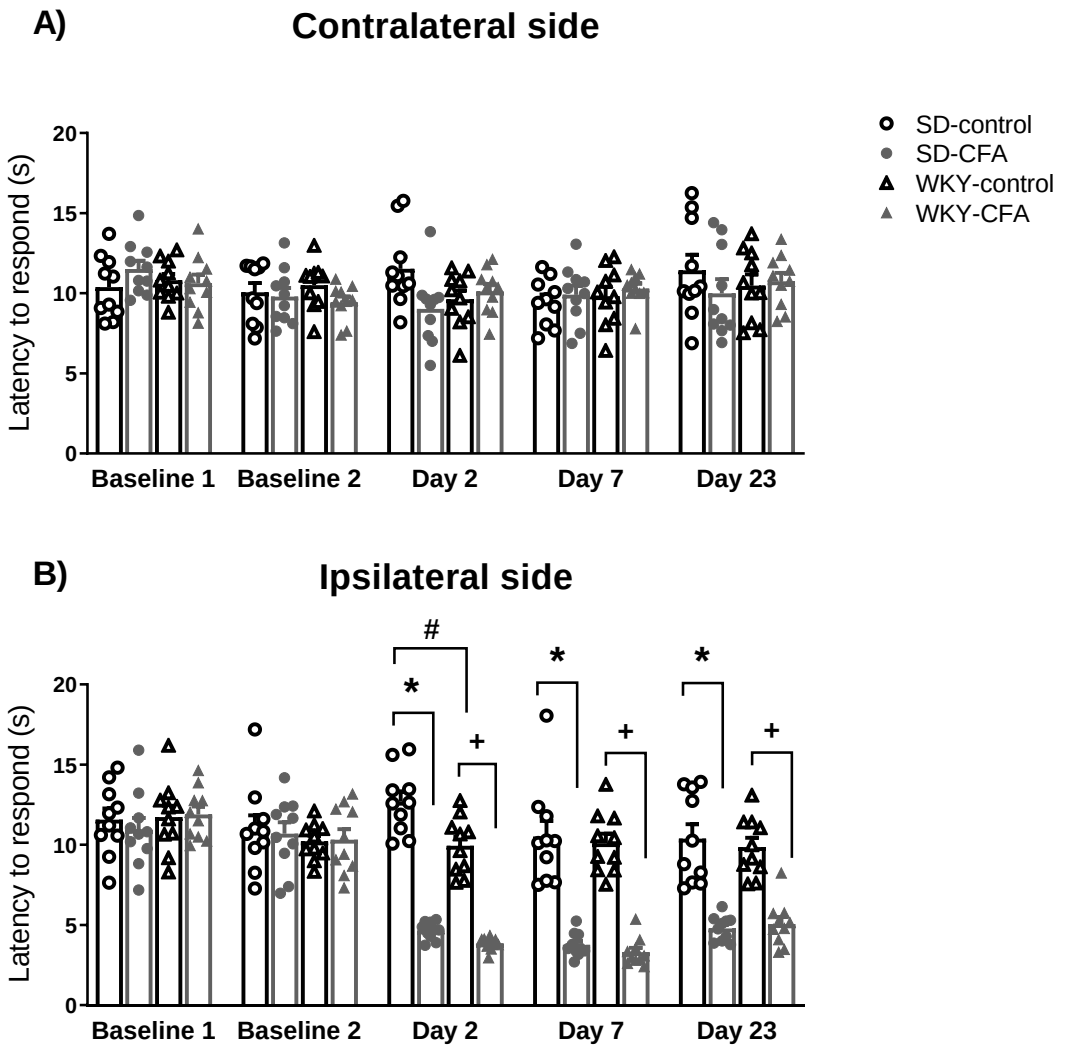


Figure 5.9: Effect of CFA injection on the withdrawal latency to noxious heat stimulus of the (A) contralateral and (B) ipsilateral hindpaw in SD and WKY rats in the HG test. Data are expressed as mean ± SEM, n=10/group. *p<0.05 (SD-CFA vs SD-control), ^{+}p<0.05 (WKY-CFA vs WKY-control), $^{\#}p$<0.05 (vs SD counterpart). CFA: complete Freund's adjuvant, HG: Hargreaves', SD: Sprague-Dawley, WKY: Wistar-Kyoto.

5.3.3. Effects of CFA injection in the PEAP in SD and WKY rats

The PEAP was employed to assess the affective component of pain in SD and WKY rats in the CFA-induced chronic inflammatory pain model. During the test, the ipsilateral paw was stimulated with a suprathreshold (60 g) VF filament when the animal was in the dark

side (preferred location) of the arena. RM ANOVA did not reveal any significant effect in tests of within-subjects effects on percentage positive response to noxious stimulus applied in the dark side. There was an overall significant effect of CFA ($F_{1,35}$=70.004, ***p*<0.001**) in tests of between-subjects effects on percentage response. As shown in Figure 5.10A, *post hoc* analysis indicated that CFA-treated SD and WKY rats showed significantly higher percentage positive response to noxious stimulation, compared to respective control counterparts, throughout the 30-min testing period [$T_{0\text{-}30}$ (in 5 min bins): SD-CFA vs SD-control, ***p*<0.05**; WKY-CFA vs WKY-control, ***p*<0.05**].

RM analysis of the time spent in the light side (Figure 5.10B) revealed a significant effect of time ($F_{3.823,133.806}$=7.170, ***p*<0.001**) in tests of within-subjects effects and an overall significant effect of strain ($F_{1,35}$=30.310, ***p*<0.001**) in tests of between-subjects effects. Further *post hoc* tests showed that the WKY-control rats spent significantly less time in the light side of the arena, compared to SD counterparts, throughout the trial [$T_{0\text{-}30}$ (in 5 min bins): WKY-control vs SD-control, ***p*<0.05**]. Moreover, neither SD nor WKY rats injected with CFA spent more time in the light side, compared to respective control counterparts, at any of the time points during the trial [$T_{0\text{-}30}$ (in 5 min bins): SD-CFA vs SD-control, $p>0.05$; WKY-CFA vs WKY-control, $p>0.05$].

All experimental groups exhibited progressive decrease in the number of entries made into the light side of the arena. RM ANOVA revealed a significant effect of time ($F_{3.379,118.263}$=55.012, ***p*<0.001**) and time x strain ($F_{3.379,118.263}$=2.657, ***p*<0.05**) in tests of within-subjects effects on number of entries into the light side (Figure 5.10C). Significant overall effects of strain ($F_{1,35}$=40.573, ***p*<0.001**), CFA ($F_{1,35}$=13.277, ***p*<0.01**), and strain x CFA interaction ($F_{1,35}$=5.232, ***p*<0.05**) were observed in tests of between-subjects effects on light side entries. *Post hoc* tests showed that WKY-control rats entered significantly less into the light side, compared to SD counterparts [$T_{0\text{-}30}$ (in 5 min bins): WKY-control vs SD-control, ***p*<0.05**] throughout the trial. Also, CFA-injected SD rats entered significantly less into the light side, compared to SD controls [$T_{0\text{-}30}$ (in 5 min bins): SD-CFA vs SD-control, ***p*<0.05**] but no such effect of CFA injection was observed in the WKY rats [$T_{0\text{-}30}$ (in 5 min bins): WKY-CFA vs WKY-control, $p>0.05$].

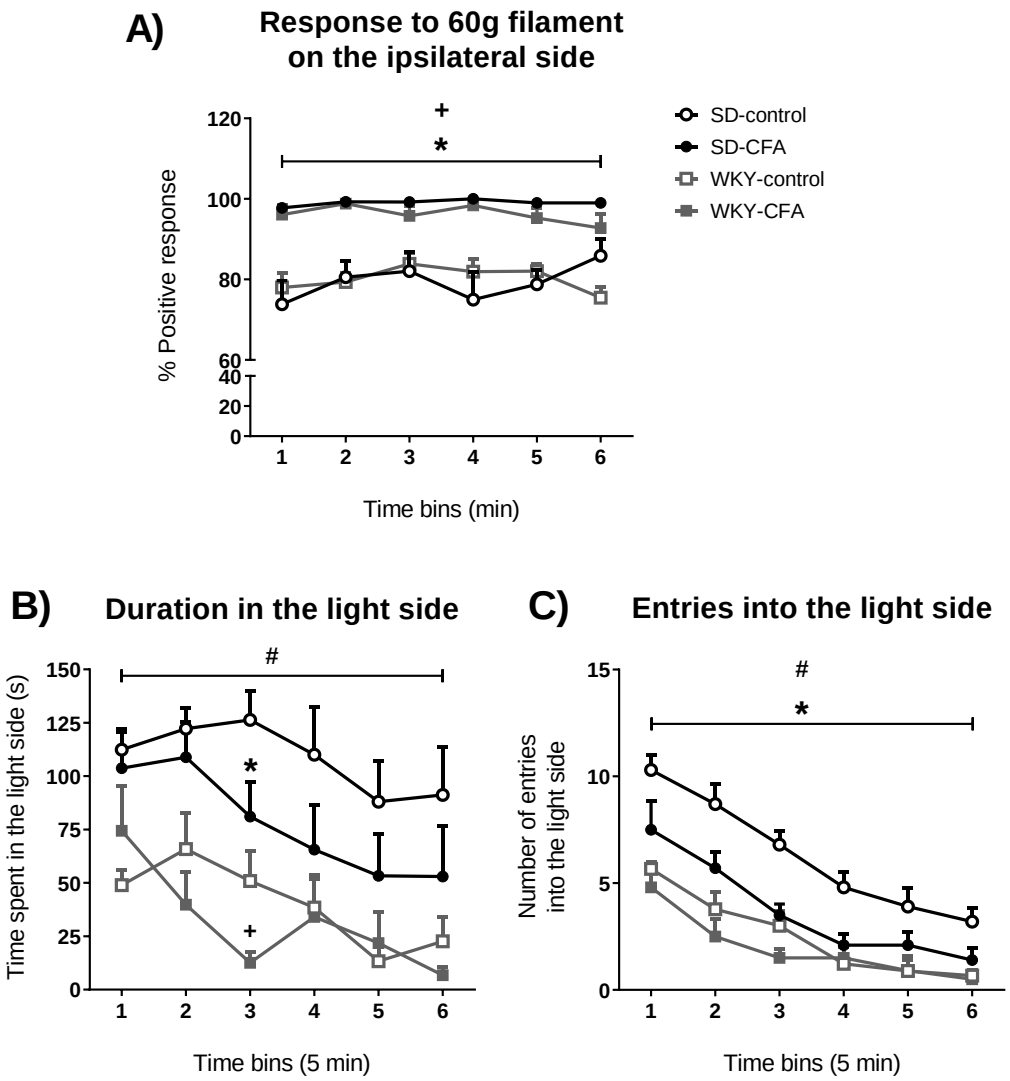


Figure 5.10: Effects of CFA injection in the PEAP in SD and WKY rats (Day 11 post-injection). The ipsilateral paw was stimulated when the animal was in the dark side and the contralateral paw was stimulated when the animal was in the light side of the arena. (A) Percentage positive response to noxious stimulation with 60 g von Frey filament on the ipsilateral paw in SD and WKY rats during the 30-min PEAP trial. Temporal profile of (B) duration and (C) number of entries in the light side of the arena in SD and WKY rats. Data are presented in 5 min bins and expressed as mean ± SEM, n = 9-10/group. *p<0.05 (SD-CFA vs SD-control); ^{+}p<0.05 (WKY-CFA vs WKY-control), $^{\#}p$<0.05 (WKY-control vs SD-control). CFA: complete Freund's adjuvant, PEAP: place escape/avoidance paradigm, SD: Sprague-Dawley, WKY: Wistar Kyoto.

5.3.4. Effects of CFA injection on anxiety-like behaviours in SD and WKY rats

On Day 13 post-CFA injection, anxiety-like behaviour was assessed in SD and WKY rats, first in the EPM test and immediately thereafter in the OF test. In the EPM, two-way ANOVA revealed that there were no significant effect of strain, CFA, or strain x CFA interaction on activities in the open arms (Time spent in open arms- strain: $F_{1,38}$=2.282, p>0.05; CFA: $F_{1,38}$=0.576, p>0.05; strain x CFA: $F_{1,38}$=0.317, p>0.05. Entries into open arms- strain: $F_{1,38}$=0.555, p>0.05; CFA: $F_{1,38}$=0.615, p>0.05; strain x CFA: $F_{1,38}$=0.301, p>0.05) (Figure 5.11A and 5.11B). A significant effect of strain ($F_{1,38}$=7.072, ***p*<0.05**), but not of CFA ($F_{1,38}$=0.054, p>0.05) or strain x CFA interaction ($F_{1,38}$=1.017, p>0.05), was observed on the time spent in closed arms but further *post hoc* SNK test revealed that there were no significant differences between relevant groups (Figure 5.11C). Two-way ANOVA revealed a significant strain x CFA interaction ($F_{1,38}$=6.570, ***p*<0.05**) on the number of entries into closed arms. *Post hoc* analysis showed there was no significant difference between relevant groups (Figure 5.11D). We also analysed the activity in the centre (connecting open and closed arms) of the EPM. Two-way ANOVA revealed a significant effect of strain only ($F_{1,38}$=29.270, ***p*<0.001**) on time spent in the centre; *post hoc* analysis revealed that WKY rats spent more time in the centre compared to SD rats (WKY-control vs SD-control, ***p*<0.05**; WKY-CFA vs SD-CFA, ***p*<0.05**; Figure 5.11E). Lastly, there was no significant ANOVA effect of strain ($F_{1,38}$=0.059, p>0.05), CFA ($F_{1,38}$=0.454, p>0.05), or strain x CFA interaction ($F_{1,38}$=2.458, p>0.05) on number of entries into the centre of EPM (Figure 5.11F).

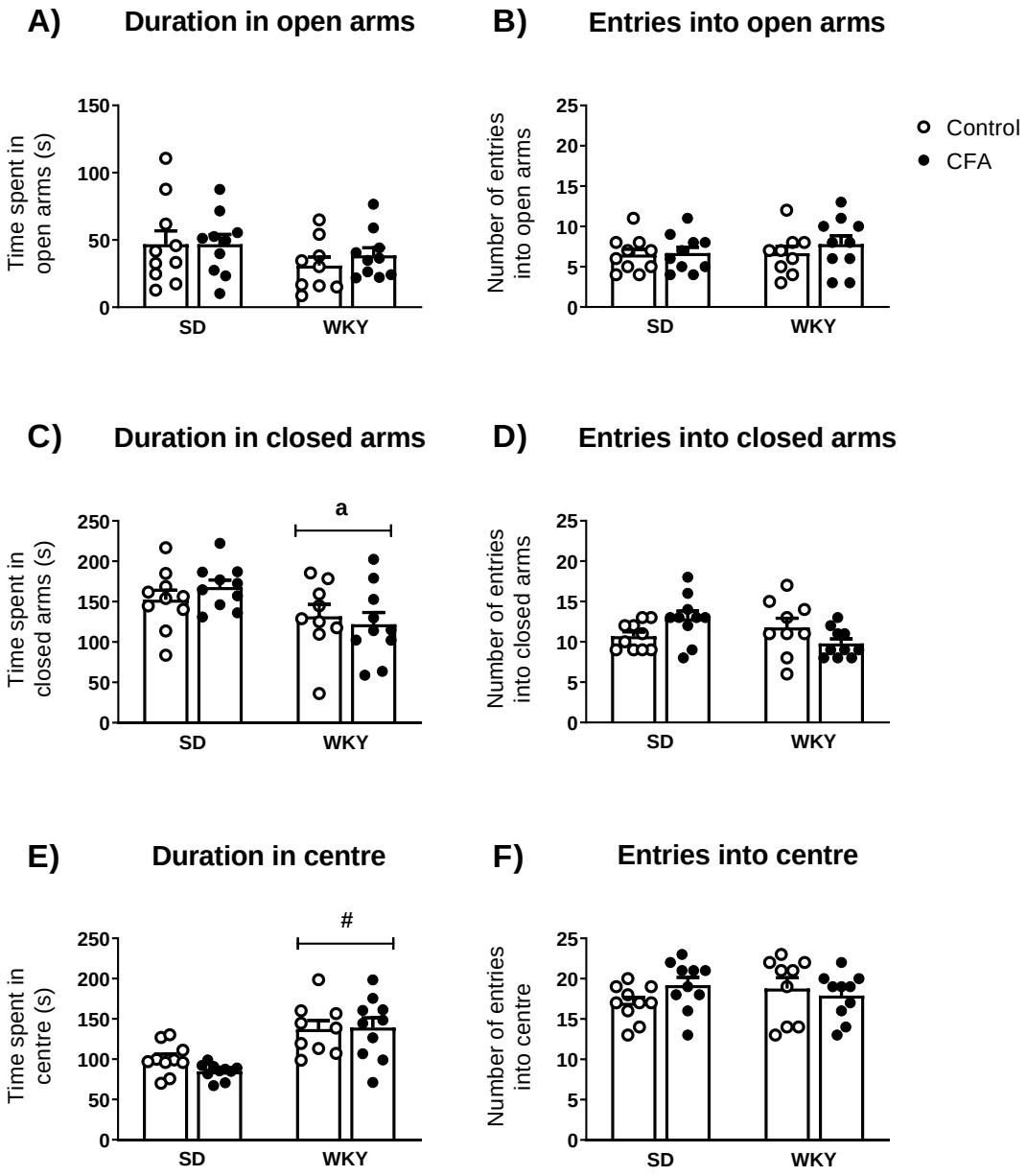


Figure 5.11: Effects of CFA injection on the duration and number of entries in (A and B) open arms, (C and D) closed arms, and (E and F) centre in the EPM test in SD and WKY rats (Day 13 post-injection). Data are expressed as mean ± SEM, n = 9-10/group. a: effect of strain (p<0.05), $^{\#}p$<0.05 (WKY vs SD counterparts). CFA: complete Freund's adjuvant, EPM: elevated plus maze, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

General locomotor activity was analysed in the OF. Two-way ANOVA revealed a significant effect of strain ($F_{1,38}$=71.284, ***p*<0.001**), but not of CFA ($F_{1,38}$=0.118, p>0.05) or strain x CFA interaction ($F_{1,38}$=2.409, p>0.05), on the distance moved in the OF arena (Figure 5.12A). *Post hoc* SNK test showed that WKY rats exhibited reduced distance moved in the OF compared to SD rats, indicating their characteristic hypolocomotor trait. Moreover, CFA injection did not affect distance moved in the arena in either strain. KW test found a significant main effect (χ^2(3)=12.650, ***p*<0.01**) on pellets excreted during the 5-min OF trial. Although WKY rats exhibited increased defaecation (number of faecal boli), *post hoc* analysis indicated there were no significant differences between the groups (Figure 5.12B). Two-way ANOVA revealed a significant effect of strain only ($F_{1,38}$=23.227, ***p*<0.001**) on the number of entries into the centre zone; *post hoc* SNK test showed WKY rats demonstrated a significant reduction in centre zone entries compared to SD counterparts (WKY-control vs SD-control, ***p*<0.05**; WKY-CFA vs SD-CFA, ***p*<0.05**; Figure 5.12C). Two-way ANOVA revealed there was no significant effect of strain ($F_{1,38}$=1.513, p>0.05), CFA ($F_{1,38}$=0.0001, p>0.05), or strain x CFA interaction ($F_{1,38}$=0.890, p>0.05) on time spent in the centre zone of the OF (Figure 5.12D). KW test showed there was no significant main effect (χ^2(3)=7.449, p>0.05) on latency to leave the centre zone of the arena (Figure 5.12E).

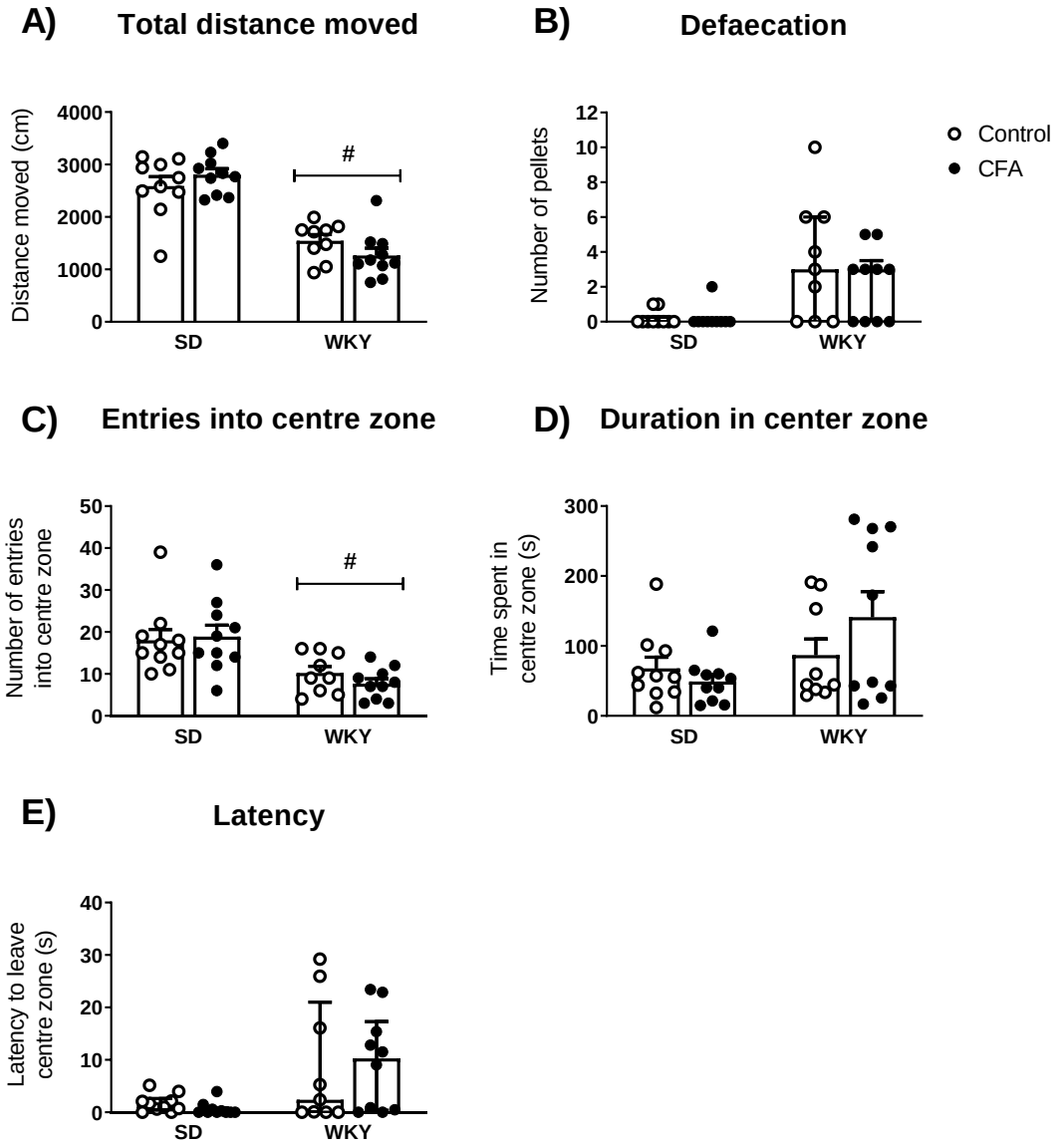


Figure 5.12: Effects of CFA injection on the (A) distance moved, (B) defaecation (number of pellets produced), (C) entries into centre zone, (D) time spent in centre zone, and (E) latency to leave centre zone in the OF test in SD and WKY rats (Day 13 post-injection). Data are expressed as (A, C, and D) mean ± SEM and (B and E) median with IQR, n = 9-10/group. $^{\#}p<0.05$ (WKY vs SD counterparts). CFA: complete Freund's adjuvant, OF: open field, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

5.3.5. Effects of CFA injection on social behaviour and memory in SD and WKY rats

Locomotor activity, measured as distance moved (cm), was analysed for each phase (habituation, sociability, and social novelty/preference) in the 3-CST (Figure 5.13A). Two-way ANOVA revealed a significant effect of strain, but not of CFA or strain x CFA interaction, in all three phases (Habituation- strain: $F_{1,39}$=22.113, ***p*<0.001**; CFA: $F_{1,39}$=1.691, p>0.05; strain x CFA: $F_{1,39}$=2.075, p>0.05. Sociability- strain: $F_{1,38}$=24.146, ***p*<0.001**; CFA: $F_{1,38}$=0.543, p>0.05; strain x CFA: $F_{1,38}$=0.065, p>0.05. Social novelty/preference- strain: $F_{1,38}$=8.464, ***p*<0.01**; CFA: $F_{1,38}$=0.492, p>0.05; strain x CFA: $F_{1,38}$=0.131, p>0.05). Further *post hoc* SNK tests showed that there were no significant differences in relevant experimental groups in the habituation and social novelty/preference trials. However, in the sociability trial, WKY rats, regardless of CFA injection, showed reduced distance moved in the arena compared to SD counterparts (WKY-control vs SD-control, ***p*<0.05**; WKY-CFA vs SD-CFA, ***p*<0.05**).

In the sociability trial, the test rat has a choice to interact with the empty cage (non-social stimulus) or the novel rat (social stimulus) (Figure 5.13B and 5.13D). There was no significant ANOVA effect of strain ($F_{1,38}$=0.008, p>0.05), CFA ($F_{1,38}$=0.542, p>0.05), or strain x CFA interaction ($F_{1,38}$=3.537, p>0.05) on time spent interacting with the empty cage (Figure 5.13B). Two-way ANOVA did reveal a significant effect of strain ($F_{1,38}$=47.328, ***p*<0.001**), but not of CFA ($F_{1,38}$=2.043, p>0.05) or strain x CFA interaction ($F_{1,38}$=1.004, p>0.05), on time spent interacting with the novel rat in the arena. *Post hoc* analysis showed that WKY rats, irrespective of CFA treatment, spent significantly less time interacting with the novel animal compared to SD counterparts in the sociability trial (WKY-control vs SD-control, ***p*<0.05**; WKY-CFA vs SD-CFA, ***p*<0.05**; Figure 5.13B). In the ANCOVA, there was a significant effect of strain on time spent interacting with the novel rat, whilst controlling for the covariate distance moved ($F_{1,38}$=24.131, ***p*<0.001**). Furthermore, in the measure of sociability index (Figure 5.13D) comparing the time spent interacting with the animal relative to empty cage, two-way ANOVA revealed a significant effect of strain only ($F_{1,38}$=4.311, ***p*<0.05**). *Post hoc* test indicated WKY-control rats spent significantly less time interacting with the novel animal compared to SD counterparts (WKY-control vs SD-control, ***p*<0.05**), suggesting a deficit in social approach behaviour in the WKY rats.

Next, in the social novelty/preference trial, the test rat is given a choice to interact with the then familiar rat or another novel rat introduced in the arena (Figure 5.13C and 5.13E). Two-way ANOVA revealed a significant effect of strain ($F_{1,38}$=14.829, ***p*<0.001**), but not of CFA ($F_{1,38}$=0.203, p>0.05) or strain x CFA interaction ($F_{1,38}$=0.687, p>0.05), on time spent interacting with the familiar rat in the arena. *Post hoc* analysis showed that WKY-control rats spent significantly less time interacting with the familiar animal compared to SD counterparts (WKY-control vs SD-control, ***p*<0.05**; Figure 5.13C). In the ANCOVA, there was a significant effect of strain on time spent interacting with the familiar rat, whilst controlling for the covariate distance moved ($F_{1,38}$=10.948, ***p*<0.01**). Two-way ANOVA also revealed a significant effect of strain ($F_{1,38}$=77.556, ***p*<0.001**), but not of CFA ($F_{1,38}$=1.603, p>0.05) or strain x CFA interaction ($F_{1,38}$=0.214, p>0.05), on time spent interacting with the novel animal in the social novelty/preference trial. Further *post hoc* SNK test indicated that WKY rats, regardless of CFA injection, spent less time interacting with the novel animal in the arena, compared to SD counterparts (WKY-control vs SD-control, ***p*<0.05**; WKY-CFA vs SD-CFA, ***p*<0.05**; Figure 5.13C). In the ANCOVA, there was a significant effect of strain on time spent interacting with the novel animal, whilst controlling for the covariate distance moved ($F_{1,38}$=58.660, ***p*<0.001**). In addition, there was no significant ANOVA effect of strain ($F_{1,38}$=2.544, p>0.05), CFA ($F_{1,38}$=0.117, p>0.05), or strain x CFA interaction ($F_{1,38}$=0.586, p>0.05) on the social novelty/preference index (Figure 5.13E).

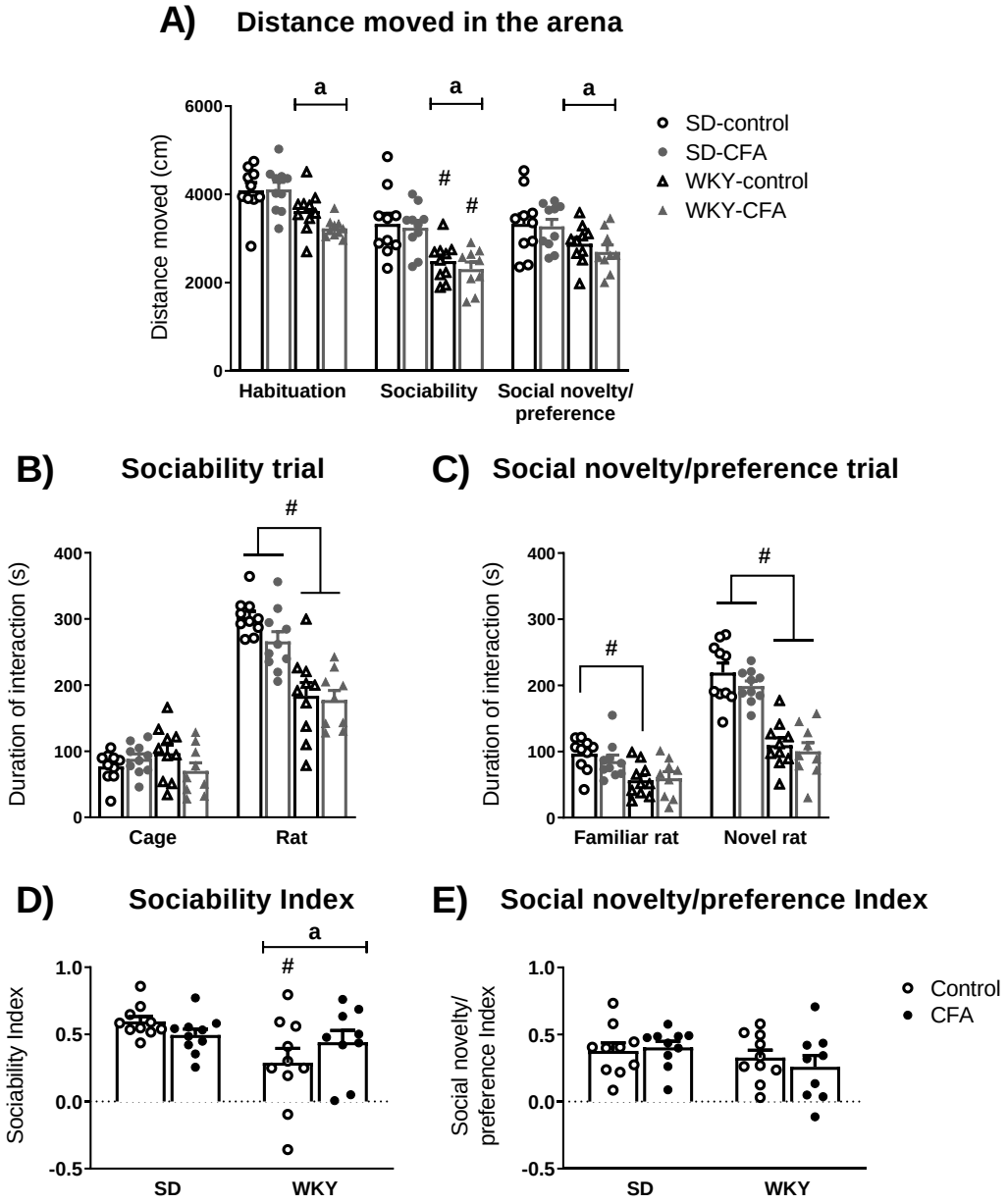


Figure 5.13: Effects of CFA injection on the social behaviour in SD and WKY rats in the 3-CST (Day 15 post-injection). (A) Distance moved in the arena in the 3 trials of 3-CST in SD and WKY rats. (B) Time spent interacting with the empty cage versus the rat during sociability trial in SD and WKY rats. (C) Time spent interacting with the familiar rat versus the novel rat during social novelty/preference trial in SD and WKY rats. Data are expressed as mean ± SEM, n = 9-10/group. a: effect of strain (habituation trial: $p<0.001$, sociability trial: $p<0.001$, social preference trial: $p<0.01$, sociability index: $p<0.05$). $^{\#}p<0.05$ (WKY vs SD counterpart). CFA: complete Freund's adjuvant, 3-CST: three-chamber sociability test, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

5.3.6. Effects of CFA injection on recognition memory in SD and WKY rats

The novel object recognition test was employed to assess any effect of CFA injection on recognition memory in the SD and WKY rats. Two-way ANOVA revealed significant effects of strain ($F_{1,35}$=26.001, ***p*<0.001**) and CFA ($F_{1,35}$=4.271, ***p*<0.05**) on time spent exploring the novel object. Further *post hoc* SNK test showed that CFA-treated SD rats spent significantly less time exploring the novel object in the arena, compared to control counterparts (SD-CFA vs SD-control, ***p*<0.05**). The WKY rats, regardless of CFA treatment, exhibited reduced duration of exploration of the novel object in comparison to SD rats (WKY-control vs SD-control, ***p*<0.05**; WKY-CFA vs SD-CFA, ***p*<0.05**; Figure 5.14A). In the ANCOVA, there was a significant effect of strain on time spent interacting with the novel object, whilst controlling for the covariate distance moved ($F_{1,35}$=5.851, ***p*<0.05**). Additionally, two-way ANOVA revealed a significant main effect of strain only ($F_{1,35}$=17.102, ***p*<0.001**) on time spent exploring the familiar objects in the arena. *Post hoc* test indicated that both control and CFA-treated WKY rats spent less time interacting with the familiar objects, compared to SD counterparts (WKY-control vs SD-control, ***p*<0.05**; WKY-CFA vs SD-CFA, ***p*<0.05**; Figure 5.14B). There was no significant effect of strain in the ANCOVA analysis, whilst controlling for distance moved ($F_{1,35}$=2.471, p>0.05).

The object exploration data were further analysed by calculating two indices: (1) preference index, which compared the time spent exploring the novel object on test day relative to the object in that location on familiarisation day, and (2) discrimination index, which compared the time spent exploring the novel object in relation to the familiar objects in the arena on the test day. Three-way ANOVA revealed significant effects of day ($F_{1,71}$=195.761, ***p*<0.001**) and strain x day interaction ($F_{1,71}$=6.907, ***p*<0.05**) on the preference index. *Post hoc* analysis showed that both SD and WKY rats spent more time exploring the novel object on the test day in comparison to the object (object 3) in the same location on familiarisation day (test day vs familiarisation day: all four experimental groups, ***p*<0.05**; Figure 5.14C), indicating that both strains preferred to interact with the novel object in that location on test day. There was no significant ANOVA effect of strain ($F_{1,35}$=0.350, p>0.05), CFA ($F_{1,35}$=0.671, p>0.05), or strain x CFA interaction ($F_{1,35}$=1.839, p>0.05) on discrimination index (Figure 5.14D).

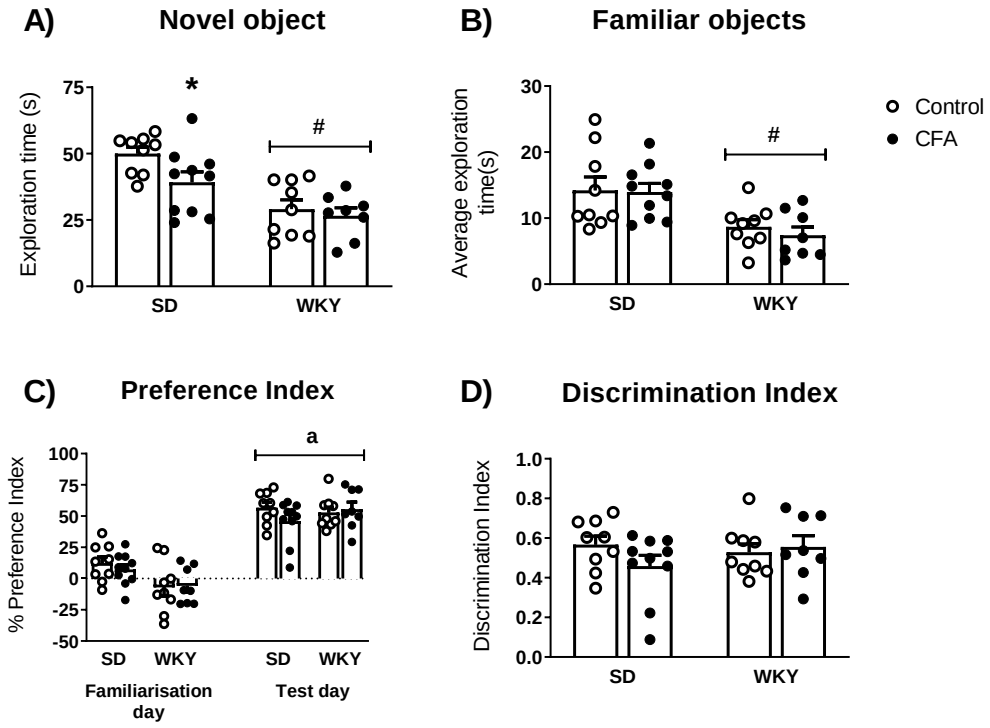


Figure 5.14: Effects of CFA injection on novel object recognition in SD and WKY rats (Day 18 post-injection). (A) Time spent exploring the novel object on the test day in SD and WKY rats. (B) Average time spent exploring the familiar objects on the test day in SD and WKY rats. (C) Percentage preference index comparing exploration time with the novel object on test day relative to object 3 on familiarisation day in SD and WKY rats. (D) Discrimination index comparing exploration time with the novel object versus the familiar objects on the test day in SD and WKY rats. Data are expressed as mean ± SEM, n = 8-10/group. a: effect of day (p<0.001). $^{\#}p$<0.05 (WKY vs SD counterparts). CFA: complete Freund's adjuvant, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

5.3.7. Effects of CFA injection on spatial memory in SD and WKY rats

Spatial memory was evaluated in SD and WKY rats using the T-maze spontaneous alternation task on Day 20-21 post-CFA injection (Figure 5.15A). Two-way ANOVA showed significant effect of strain ($F_{1,39}$=36.167, ***p*<0.001**), but not of CFA ($F_{1,39}$=0.447, p>0.05) or strain x CFA interaction ($F_{1,39}$=0.251, p>0.05), on percentage alternation (correct arm choice). *Post hoc* SNK test showed that WKY rats, irrespective of CFA injection, exhibited significantly lower alternation of arms in the choice phase, compared

to SD counterparts. There were no effects of strain ($F_{1,39}$=0.000, p>0.05), CFA ($F_{1,39}$=0.000, p>0.05), or strain x CFA interaction ($F_{1,39}$=2.800, p>0.05) on percentage error in alternation (incorrect arm choice). Two-way ANOVA revealed significant effect of strain ($F_{1,39}$=26.036, ***p*<0.001**), but not of CFA ($F_{1,39}$=0.321, p>0.05) or strain x CFA interaction ($F_{1,39}$=2.009, p>0.05), on percentage omission of trials (failure to choose an arm). *Post hoc* analysis indicated that WKY rats, regardless of CFA injection, failed higher number of times in choosing an arm, compared to SD rats. Furthermore, two-way ANOVA revealed a significant effect of strain only ($F_{1,39}$=13.322, ***p*<0.01**) on latency to choose a goal arm in the sample phase. *Post hoc* test showed that both control and CFA-treated WKY rats took significantly longer to enter into an arm in comparison to SD counterparts (Figure 5.15B).

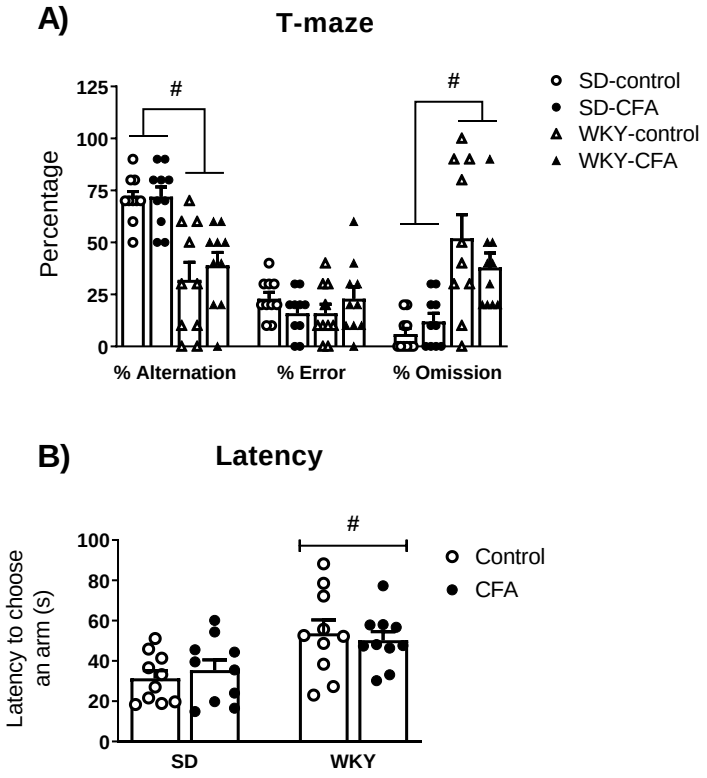


Figure 5.15: Effect of CFA injection on the spatial memory in SD and WKY rats (Days 20 and 21 post-injection) in the T-maze spontaneous alternation test. (A) Percentages of spontaneous alternation, errors in alternation, and omission of trials in the T-maze in SD and WKY rats. (B) Average latency to choose an arm in the sample phase in the T-maze alternation task in SD and WKY rats. Data are expressed as mean ± SEM, n = 10/group. $^{\#}p$<0.05 (WKY vs SD counterparts). CFA: complete Freund's adjuvant, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

5.4. Discussion

The study described in this chapter assessed sensory and affective dimensions after inducing chronic inflammatory pain using CFA in WKY versus SD rats. In addition, we examined CFA-induced development of anxiety-like behaviour and cognitive impairment in the two rat strains, and if these affective and cognitive deficits were enhanced in the WKY rats, in comparison to SD counterparts. Both SD and WKY rats showed CFA-induced mechanical allodynia and thermal hyperalgesia; however, CFA-injected WKY rats displayed enhanced mechanical allodynia, compared to SD counterparts, at all post-injection time points. In the PEAP, CFA-injected WKY and SD rats did not avoid the dark (preferred) side of the arena following stimulation of the ipsilateral paw. Neither strain exhibited any CFA-induced anxiety-like behaviour in the EPM and OF tests at the time point tested. Overall, compared to SD rats, WKY rats displayed impairments in social behaviour and in spatial and recognition memory. CFA-induced chronic inflammatory pain did not affect social behaviour and spatial memory in either strain at the time points tested. A reduction in novel object exploration was observed in the CFA-injected SD rats, but not in the WKY counterparts, in the NOR test.

In the present study, WKY rats displayed increased sensitivity (lower paw withdrawal threshold) to mechanical stimulation, compared to SD counterparts, at pre-CFA time point (baseline 2) and on the contralateral (non-CFA injected) side. Previous studies have reported similar allodynic response to mechanical stimulation in naive WKY rats, in comparison to SD rats (Taylor et al., 2001; Hestehave et al., 2019a). Interestingly, CFA-injected WKY rats developed enhanced mechanical allodynia, compared to SD counterparts. A recent study has compared nociceptive responses to mechanical stimulation in WKY and SD rats in the CFA model (Hestehave et al., 2019b). However, the authors reported that the WKY rats developed a less robust mechanical hypersensitivity than SD rats after CFA injection. Discrepancy between the studies could be due to the different pain tests used (von Frey versus Randall Selitto). With regards to thermal hyperalgesia following CFA injection, both strains developed similar hypersensitivity to noxious heat stimulus. Hence, these results indicate that (in the measures of sensory dimensions of pain) the WKY rats exhibit exacerbated mechanical allodynia, but not enhanced thermal hyperalgesia, in the CFA-induced chronic inflammatory pain model.

The experience of pain is multidimensional comprising of both sensory and affective components that are mediated by overlapping neural circuitry. The present study also examined the affective dimension of pain in SD and WKY rats using PEAP. This paradigm is based on a conflict between the rodents' natural tendency to avoid light on one hand and the avoidance of noxious stimuli in the dark (preferred) location on the other hand (Fuchs and McNabb, 2012). Studies in inflammatory pain models have shown that rodents spend more time in the light area, compared with control counterparts, reflecting increased aversive quality of the stimulation of the injured paw (Labuda and Fuchs, 2000, 2001; Uhelski and Fuchs, 2009; Boyce-Rustay et al., 2010; Refsgaard et al., 2016). In our study, CFA-injected SD and WKY rats, compared to control counterparts, exhibited higher response to a suprathreshold mechanical stimulation on the ipsilateral paw when in the dark side, indicating increased CFA-induced sensitivity of the injured paw in both strains. However, in contrast to previous reports (Labuda and Fuchs, 2000; Boyce-Rustay et al., 2010; Refsgaard et al., 2016), the CFA-injected SD rats preferred the dark side over the course of the trial, compared to control counterparts. Methodological difference relating to time point of PEAP testing (relative to CFA injection) in the CFA model between our study and those published may partly explain this discrepancy. Previous studies performed PEAP test at early time points (24-48 h) following induction of inflammatory state whereas we conducted the test on Day 11 post-CFA injection. Thus, it appears that although CFA-induced enhanced sensory response to noxious mechanical stimulation persists, the affective dimension of pain may differ over time, possibly due to compensatory or maladaptive responses in an on-going pain state. A recent study has examined the development of pain aversion and pain-induced anxiety in SD rats in the CFA model (Wu et al., 2017). The authors found that CFA induced aversion in a place conditioning paradigm, which gradually attenuated over 4 weeks. On the other hand, anxiety-like behaviour in the elevated zero maze was only apparent on Day 28 post-CFA injection and not in the early time points. This further supports the contention that manifestation of the negative affective component of pain may follow different timecourses.

Our study is the first to investigate the affective component of pain using PEAP in WKY rats in the CFA model. Overall, the WKY rats displayed reduced activity on the light side, compared to SD rats, both in terms of time spent and entries into the light side. WKY rats display reduced locomotor activity in the OF (seen in this study also), compared to other

rat strains (Paré, 1989b; Drolet et al., 2002; Pardon et al., 2002; Burke et al., 2010, 2016). In addition, WKY rats exhibit a behaviourally inhibited phenotype (Paré, 1994; McAuley et al., 2009), which is considered as a risk factor for anxiety disorders in humans (Lahat et al., 2011). Thus, the elevated anxiety level along with diminished motor activity of the WKY rats may, to some extent, account for their behaviour observed in PEAP test in the current study.

In the EPM and OF tests to evaluate anxiety-related behaviour, the WKY rats displayed an overall propensity to escape/avoidant behaviours in novel environments, compared to SD rats. We note that the WKY rats in the present study did not display classic anxiety-like behaviour for some of the anxiety-related measures in these tests (such as time spent in the open arms of the EPM or in the centre zone of the OF), as reported previously (Gentsch et al., 1987; Paré, 1992; Tejani-Butt et al., 1994; Malkesman and Weller, 2009; Burke et al., 2010, 2016). However, in comparison to SD counterparts, WKY rats spent significantly more time in the centre of the EPM, an area that connects the open and closed arms of the maze. Such behaviour has been interpreted in previous studies as a tendency of the WKY rats to be more ambivalent or indecisive (Nosek et al., 2008; Nam et al., 2014), a trait observed in affective disorders in humans (Uher et al., 2014). Moreover, in accordance with previous studies (Paré, 1989b; Drolet et al., 2002; Pardon et al., 2002; Burke et al., 2010, 2016), the WKY rats exhibited decreased activity (distance moved and entry into the centre zone) in the novel aversive environment of the OF, indicating a certain degree of behavioural inhibition, and perhaps elevated fear/anxiety level.

Our study did not find any CFA-induced anxiety-like behaviours in SD or WKY rats. This is not consistent with previous studies showing enhanced anxiety-like behaviours in rats in the CFA model of chronic inflammatory pain (Parent et al., 2012; Wu et al., 2017; Zhong et al., 2019). However, in these reports, anxiety tests (EPM, OF, light/dark box, elevated zero maze) were performed 3-4 weeks after CFA injection into the hindpaw of the rats as opposed to Day 13 in our study. This methodological difference in testing time point may account for the discrepant results here. Studies in mice have reported CFA-induced anxiety-like behaviour at early (1-7 days) (Chen et al., 2013; Refsgaard et al., 2016; Yue et al., 2018), and later (21-28 days) (Narita et al., 2006a; Wang et al., 2015a; Guo et al., 2016) time points relative to intraplantar injection of CFA, although some studies did not find any effect of CFA on anxiety at these corresponding time points (Urban et al., 2011; Sheahan et al., 2017). The use of different strains of mice, anxiety

tests, and uni- or bilateral CFA injection into the hindpaws may account for these variable reports. Taken together, these studies indicate that CFA-induced manifestation of anxiety-like behaviours may follow different timecourse particularly depending on the species used to model inflammatory pain state.

The present study also assessed different cognitive domains, namely social approach and social memory, recognition memory, and spatial memory, in SD and WKY rats in the CFA-induced chronic inflammatory pain model. In the sociability trial of 3-CST, WKY rats, compared to SD counterparts, spent significantly less time interacting with the novel stimulus rat relative to the empty cage (observed both in duration of interaction and sociability index). Such inhibition to social cues in the WKY rats has been demonstrated in other studies (Ferguson and Cada, 2004; Nam et al., 2014). In addition, the WKY rats spent less time interacting with the novel rat in social novelty/preference trial, compared to SD counterparts. However, no difference was observed between WKY and SD rats in the relative time spent interacting with novel versus familiar rat, as measured by social novelty/preference index. ANCOVA suggested that these differences in interaction times between the two strains in sociability and social preference phases are unlikely to be due to strain-related differences in locomotor activity. The reduced social interaction time displayed by the WKY rats is likely related to its social avoidance behaviour, rather than a deficit in social recognition memory (Zhang-James et al., 2014). Next, in the test of object learning and recognition memory, WKY rats spent less time exploring the novel object, compared to SD counterparts. ANCOVA showed that this difference between the strains in novel object interaction time is unlikely to be due to strain-related differences in locomotor activity. Our findings are similar to previous studies that reported reduced novel object exploration by the WKY rats, compared to Wistar counterparts (Langen and Dost, 2011; Shoval et al., 2016; Willner et al., 2019), suggesting an impairment in recognition memory in the WKY rats. There was no difference in discrimination or preference indices between the two strains. So alternatively the WKY rats may not have a deficit in recognition memory *per se* but exhibit an overall avoidance behaviour to novelty (Delini-Stula and Hunn, 1985) that is reflected in reduced object exploration times. In the T-maze test for evaluating spatial memory, the WKY rats exhibited reduced alternation of the goal arm, compared to SD rats. This reduced alternation might be due to their increased failure to choose an arm since no strain difference was observed in making incorrect arm choice (error in alternation). Though no other study to date has

tested the WKY rats in the T-maze, previous studies have reported deficit in spatial learning and memory in the WKY rats in the Morris water maze, compared to SD counterparts (Grauer and Kapon, 1993; Wyss et al., 2000; Doharty, 2018). The indecisive nature of the WKY rats is further reflected (Nosek et al., 2008; Nam et al., 2014) in the T-maze task where the WKY rats failed to choose an arm in more number of occasions than SD counterparts. It should be noted that the cognitive tasks used here have a pronounced locomotor component and so the results of these tests may be influenced given the WKY rats demonstrate hypolocomotor activity (Paré, 1989a; Pardon et al., 2002; Burke et al., 2010). Interestingly, WKY rats are not hypolocomotive in home cage activity (Burke et al., 2016) or in the rotarod test (Ferguson et al., 2003). This suggests that the WKY rats may not have an overt deficit in motor activity, but their deficiencies are unmasked in a novel aversive environment. Hence, further studies are needed to investigate the deficits in different cognitive domains in the WKY rats and the relative influence of their behavioural phenotype on cognitive impairment.

Neither SD nor WKY rats receiving intraplantar CFA injection exhibited impairment in social recognition and spatial memory in the present study. Reduction in active social interaction has been observed in rats on Day 28 following intraplantar CFA injection (Parent et al., 2012). In addition, CFA-induced impairment in spatial learning and memory in rats has been reported previously (Li et al., 2005; Grégoire et al., 2014). However, differences in behavioural tests used (e.g. Y-maze, Morris water maze) and time points of testing (Day 28 vs Day 15 in assessing social recognition memory) in these published reports may account for the discrepant results observed in our study. In the NOR test, CFA induced a significant, albeit modest, reduction in the exploration of the novel object (without affecting exploration of the familiar objects) in the SD rats, but not in the WKY counterparts. A study by Amaral and coworkers demonstrates that intraplantar CFA injections on postnatal day 1 in rats impaired cognitive performance in the NOR test when assessed on postnatal day 60 (Amaral et al., 2015). Moreover, other studies have reported CFA-induced cognitive impairment in rats in the delayed nonmatching-to-position lever-press task, a behavioural paradigm that sensitive to deficits in learning, memory, and attention (Cain et al., 1997). Hence, there is a possibility that if we performed NOR test at a later time point than that in the present study, we may observe greater cognitive impairment in the SD rats. This notion was further explored in a follow-up study in the next chapter (Chapter 6).

In summary, the present study further characterised the CFA-induced chronic inflammatory pain model in SD and WKY rats. The results indicate exacerbated mechanical allodynia, but not heat hyperalgesia, in the WKY versus SD rats in the CFA model. Moreover, the data from the PEAP test showed that manifestation of the affective component of pain may follow a different timecourse than the sensory component. Apparent differences exist in the domains of social behaviour and recognition and spatial memory between WKY and SD rats. The behaviourally inhibited trait of the WKY rats may affect performance in some of these cognitive parameters. Our study did not provide evidence for CFA-induced anxiety-like behaviour and impairments in social or spatial memory in either strain at the time points tested. Only a modest deficit in recognition memory was observed in the SD rats in the CFA model. To conclude, this study extended the literature on behavioural characteristics of the WKY rat strain as well as CFA-associated effects on different behavioural domains (pain, affect, and cognition) in both SD and WKY rats.

6. Chapter 6: Effects of modulating kappa- and delta-opioid receptors on pain-, motivation-, and cognition-related behaviours in the rat complete Freund's adjuvant model of chronic inflammatory pain

6.1. Introduction

Chronic pain is an enormous global health problem, resulting in emotional distress and functional disability and imposing huge socioeconomic burden. Although mounting research over the last few decades has advanced our understanding of the neurobiological mechanisms underlying different types of chronic pain conditions, the therapeutic strategies against chronic pain are currently limited and opioids are still one of the most commonly indicated drugs here. However, the vast majority of the opioids used clinically act primarily through the MOP, producing potent analgesia along with deleterious adverse effects (including constipation, respiratory depression, tolerance, and addiction) that severely limit their therapeutic use. In addition, there is high prevalence of affective disorders (anxiety and depression) (Asmundson and Katz, 2009; Scott et al., 2016; Stubbs et al., 2016) and cognitive impairment (Moriarty et al., 2011; Shin et al., 2012) with chronic pain, and this co-morbidity poses an additional challenge to the management of chronic pain. In this regard, other members of the classical opioid receptor family, the kappa (KOP)- and delta (DOP)-opioid receptors, have gained much attention as potential alternatives to MOP-based therapy.

The DOP are expressed in both the central and peripheral nervous systems, including Aδ- and C-fibre nociceptive neurons, DRG, spinal cord, cortex, amygdala, striatum, and hippocampus – regions that are implicated in processing pain, emotion, and cognition (Moskowitz and Goodman, 1984; Lever et al., 1992; Mansour et al., 1995; Scherrer et al., 2006; Le Merrer et al., 2009). Interestingly, DOP expression is low in the mid-brain (e.g. PAG) and brainstem (e.g. nucleus raphe magnus) (Le Merrer et al., 2009). In tests of acute pain, pharmacological activation of DOP produces weak antinociception (Gallantine and Meer, 2005). However, DOP agonists produce potent antinociception in rodent models of persistent or chronic pain (Stewart and Hammond, 1994; Fraser et al., 2000; Mika et al., 2001; Holdridge and Cahill, 2007), including the chronic Freund's adjuvant (CFA)

model of inflammatory pain (Hurley and Hammond, 2000; Cahill et al., 2003; Codd et al., 2009). Such enhanced efficacy of DOP agonists is partly due to upregulation of DOP trafficking and expression on the cell surface membrane (Cahill et al., 2003; Commons, 2003; Kabli and Cahill, 2007) and increased functional activity (Pradhan et al., 2013) in chronic pain states. In addition, DOP knockout mice show no (or only subtle) alterations in nociceptive thresholds in acute pain models (e.g. hot plate, tail withdrawal, and formalin tests) (Filliol et al., 2000; Martin et al., 2003) but exhibit augmented thermal and mechanical sensitivity in models of chronic inflammatory (Gavériaux-Ruff et al., 2008, 2011) and neuropathic pain (Nadal et al., 2006), compared to wild-type counterparts. These pharmacological and genetic findings suggest a key role for DOP in alleviating chronic pain states.

Additionally, the DOP plays a prominent role in modulating emotional responses. Genetic deletion of the DOP increases immobility in the forced swim test in mice (Filliol et al., 2000). In contrast, both the administration of enkephalinase inhibitors (which increase synaptic level of endogenous DOP preferential peptide, enkephalin) (Jutkiewicz et al., 2006; Yang et al., 2011) as well as selective DOP agonists (Broom et al., 2002; Jutkiewicz et al., 2004; Saitoh et al., 2004, 2008, 2011; Torregrossa et al., 2006; Naidu et al., 2007; Vergura et al., 2008; Nozaki et al., 2014) reduce despair-like behaviour in rodents in several models of depression. Furthermore, pharmacological data suggest that the DOP modulate learning and memory processes, although the exact implication of activating or inhibiting DOP activity on memory performance remains unclear owing to conflicting results (for review see Pellissier et al., 2018). Nevertheless, constitutive DOP knockout mice display impairment in object location learning and short-term memory (Le Merrer et al., 2013), indicating a role of DOP in cognitive performance. Hence, the DOP system appears to be a potential therapeutic target in comorbid chronic pain and affective disorders and/or cognitive impairment.

In comparison to DOP, the KOP shows overlapping but also distinct distribution in neural circuits of pain, affect, and cognition (Minami et al., 1993; Depaoli et al., 1994; Gutstein et al., 1998; Snyder et al., 2018). As highlighted in Chapter 4, activation of central KOP produces antinociception but also induces negative affective states (e.g. dysphoria, aversion, anhedonia, and behavioural despair) in humans (Pfeiffer et al., 1986) and rodents (Shippenberg and Herz, 1987; Mague et al., 2003; Todtenkopf et al., 2004; Carlezon et al., 2006; Bruchas et al., 2007; Land et al., 2008). On the other hand,

pharmacological blockade of KOP produces antidepressant-like effects in a number of rodent models, including the forced swim (Mague et al., 2003; Beardsley et al., 2005; Zhang et al., 2007; Reindl et al., 2008; Rorick-Kehn et al., 2014), learned helplessness (Newton et al., 2002; Shirayama et al., 2004), and social defeat (Grimwood et al., 2011) tests. Moreover, recent studies have shown that blocking KOP with nor-BNI or JDTic attenuates chronic inflammatory pain-induced tonic-aversion (Liu et al., 2019b) and anhedonia (Massaly et al., 2019) in rodents, suggesting that the KOP antagonists may be useful in alleviating ongoing pain states and pain-induced negative affect (Xie et al., 2017; Navratilova et al., 2019). With regard to cognition, direct administration of the KOP agonist U50488 into the hippocampus impairs performance in contextual fear conditioning and water maze tasks in mice, which is blocked by the antagonist nor-BNI (Daumas et al., 2007). Furthermore, nor-BNI reverses dynorphin B- or stress-induced impairment in spatial (Sandin et al., 1998) and recognition (Carey et al., 2009) memory in rodents, indicating KOP blockade may prevent cognitive deficit. Taken together, these studies indicate potential utility of KOP antagonists in chronic pain and associated emotional disorders and/or cognitive dysfunction. Indeed, a recent study has reported that disrupting the KOP gene in a mouse model of osteoarthritis enhances mechanical allodynia and pain-induced anhedonia but reduces deficit in object recognition memory (Negrete et al., 2017), indicating a complex role of the KOP in the modulation of pain and associated cognitive impairment.

In addition to exploring opioid receptors other than the MOP for improved therapeutics, others have proposed targeting multiple opioid receptors to improve/maintain the efficacy of opioid modulation while reducing the adverse effect profile. In recent times, the notion of combining two opioid modulatory drugs to produce a certain opioid pharmacological profile (favouring the desirable behavioural response and minimising side effects) has gained much attention (Almatroudi et al., 2015; Burke et al., 2019). A pervious study has reported reduced DOP but enhanced KOP activity (measured using agonist-induced [^{35}S]GTPγ binding assay) in the amygdalar membranes in a mouse model of chronic inflammatory pain exhibiting comorbid anxiety (Narita et al., 2006a). The amygdala plays a key role in nociceptive and affective modulation and memory encoding (Baxter and Murray, 2002; Bushnell et al., 2013). The authors have suggested that these changes in the functional activity of DOP and KOP possibly contribute to chronic pain-induced emotional and/or cognitive dysfunction. Hence, given the pharmacological profiles of

DOP and KOP, a combination drug approach with a DOP agonist and a KOP antagonist may be useful in chronic pain and comorbid conditions.

In the previous chapter (Chapter 5), we demonstrated development and persistence of mechanical allodynia and heat hyperalgesia for 3 weeks in a rat model of CFA-induced chronic inflammatory pain, in accordance with published reports (Hargreaves et al., 1988; Stein et al., 1988; Chaplan et al., 1994; Soignier et al., 2011; Moriarty et al., 2012). However, our study did not find any CFA-induced manifestation of negative affective state, possibly due to the time point of testing used [Day 13 post-injection compared to Day 21 or 28 in previous studies (Parent et al., 2012; Wu et al., 2017; Zhong et al., 2019)]. We also observed a modest CFA-induced impairment in object learning and recognition memory in SD rats on Day 18 post-injection. Therefore, the present chapter aims to further investigate the effects of CFA-induced chronic inflammation on manifestation of negative affect- and cognition-related behaviours over time in SD rats. In addition, we hypothesised that chronic administration of a DOP agonist (SNC80) and/or a KOP antagonist (DIPPA) will alleviate enhanced nociceptive responding and any negative affect- and/or cognition-related deficits induced by CFA. The specific aims of the work described in this chapter are as follows:

- To assess the effects of chronic systemic administration of SNC80 and DIPPA on mechanical allodynia and heat hyperalgesia induced by CFA injection.
- To assess recognition memory and motivation-related behaviour at two time points post-CFA injection.
- To determine the effects of chronic systemic administration of SNC80 and DIPPA, alone or in combination, on recognition memory in control and CFA-injected rats.
- To determine the effects of chronic systemic administration of SNC80 and DIPPA, alone or in combination, on motivation-related behaviour (anhedonia) in control and CFA-injected rats.

6.2. Materials and methods

6.2.1. Animals

Experiments were carried out in male SD rats (7-8 weeks old, 170-220 g on arrival; Envigo, Bicester, UK). Rats were maintained under a standard 12:12 h light/dark cycle (lights on from 07:00-19:00 h) in a temperature- (21±2°C) and humidity-controlled (45-55%) room throughout the study. Upon arrival, all animals were housed in groups of 3-4 per cage in plastic bottom cages (45x20x20 cm^3) containing 3Rs basic bedding (>99% recycled paper; 3Rs Lab, Fibrecycle Ltd, North Lincolnshire, UK) and sizzle nest material (LBS Biotechnology, Horley, UK). Following 5 days of acclimatisation to the unit, animals were housed singly in cages for the rest of the study. Food (14% protein rodent diet; Envigo, Bicester, UK) and water were available *ad libitum*. All experimental procedures were carried out during the light phase between 08:00 and 18:00 h. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway. The work was carried out under license from the Health Products Regulatory Authority in the Republic of Ireland and in accordance with EU Directive 2010/63. The study was also designed and carried out in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

6.2.2. Complete Freund's adjuvant injection

Immunogenic complete Freund's adjuvant (CFA; Sigma-Aldrich, Wicklow, Ireland), consisting of heat-killed and dried *Mycobacterium tuberculosis* in an 85% paraffin oil and 15% mannide monooleate suspension, was used to induce a chronic inflammatory pain state as described previously in Chapter 5 (section 5.2.2). On Day 0, rats were injected once with 100 µL CFA (1 mg/mL) in the plantar surface of the right hindpaw under brief isoflurane anaesthesia (3% in 0.8 L/min O_2). Control (non-CFA) rats received an intraplantar needle insertion into the right hindpaw under the same conditions. After a brief recovery period, animals were returned to their home cages.

6.2.3. Chemicals and drug preparation

The DOP agonist SNC80 [(+)-4-{(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl}-*N*,*N*-diethylbenzamide; Tocris, Bristol, UK] was dissolved in 0.01 M HCl/0.89% w/v NaCl. The KOP antagonist DIPPA hydrochloride [2-(3,4-Dichlorophenyl)-N-methyl-N-{(1S)-1-(3-isothiocyanatophenyl)-2-(1-

pyrrolidinyl)ethyl}acetamide hydrochloride; Tocris, Bristol, UK] was dissolved in 20% dimethyl sulphoxide (DMSO; Sigma-Aldrich, Wicklow, Ireland) in sterile deionised water. The final doses of the drugs were corrected for the salt form and given as mg free base equivalent per kg body weight. Both drugs were administered once daily via the subcutaneous (s.c.) route (injection volume: 1 mg/mL) for 30 days. The doses and time points of drug administration were chosen based on previous studies (Chang et al., 1994b; Fraser et al., 2000; Gallantine and Meer, 2005; Carr and Lucki, 2010; Carr et al., 2010; Gavériaux-Ruff et al., 2011; McHugh, 2019). DIPPA and SNC80 were always administered 24 h and 30 min, respectively, prior to time point of behavioural testing (except for that of splash test which was conducted immediately after test session of NOR task). Hence, DIPPA dosing began on Day 0 (24 h before behavioural testing on Day 1) and continued until Day 29. Similarly, SNC80 dosing started on Day 1 (30 min before behavioural testing) and continued until Day 30. In this way the acute component of drug effect was also investigated to assess any development of drug tolerance. For the splash test, 10% (w/v) sucrose (Fisher Scientific, Leicestershire, UK) solution was freshly prepared on the test day.

6.2.4. Study design

The experimental timeline is illustrated in Figure 6.1. Two sets of baselines were conducted for nociceptive responses to cutaneous mechanical and thermal stimuli. Baseline paw withdrawal threshold to mechanical stimulus in the von Frey test were determined 5 and 2 days before CFA injection. Similarly, baseline paw withdrawal latencies to noxious heat stimulus in the Hargreaves' test were measured 4 and 1 days prior to CFA injection. On Day 0, animals were randomly assigned to receive control/CFA injection and then to drug treatment groups (see Table 6.1 for experimental groups and n numbers). Rats were administered once daily with vehicle, SNC80 (1 mg/kg, s.c.), DIPPA (1 mg/kg, s.c.), or combination of SNC80 and DIPPA (each 1 mg/kg, s.c.) for 30 days. Each animal received two injections to control for the vehicles used and times of administration. Also, the sequence of treatments was randomised to control for the order of testing and time of the day. The animals were examined in a series of behavioural investigations: (1) mechanical allodynia was assessed using von Frey test on Days 1, 7, 21, and 28; (2) thermal hyperalgesia was determined using Hargreaves' test on Days 2, 8, 22, and 29; (3) object learning and long-term recognition memory was evaluated in the novel object recognition test at two time points, Day 16-18 and Day 25-27; and (4)

anhedonia (motivation-related behaviour) was assessed in the splash test also at two time points, Day 18 and Day 27. On Day 30, rats were euthanised by decapitation.

Table 6.1: Summary of experimental groups. CFA: complete Freund's adjuvant, i.pl.: intraplantar, n: number, q.d.: once daily, s.c.: subcutaneous.

Group	Control/CFA (i.pl.) on Day 0	Treatment (s.c., q.d., 30 days)	n
1	Non-CFA	Vehicle	10
2	Non-CFA	SNC80 1 mg/kg	10
3	Non-CFA	DIPPA 1 mg/kg	10
4	Non-CFA	SNC80 1 mg/kg + DIPPA 1 mg/kg	10
5	CFA	Vehicle	10
6	CFA	SNC80 1 mg/kg	10
7	CFA	DIPPA 1 mg/kg	10
8	CFA	SNC80 1 mg/kg + DIPPA 1 mg/kg	10

6.2.5. Behavioural testing

6.2.5.1. Sensory testing

6.2.5.1.1. von Frey test for mechanical allodynia

The von Frey (VF) test to assess mechanical allodynia was performed on Days 1, 7, 21, and 28 post CFA-injection using the up-down Dixon method as described previously in Chapter 5 (section 5.2.4.1.1).

6.2.5.1.2. Hargreaves' test for thermal hyperalgesia

The Hargreaves' (HG) test to assess heat hyperalgesia was performed on Days 2, 8, 22, and 29 post CFA-injection as described previously in Chapter 5 (section 5.2.4.1.2).

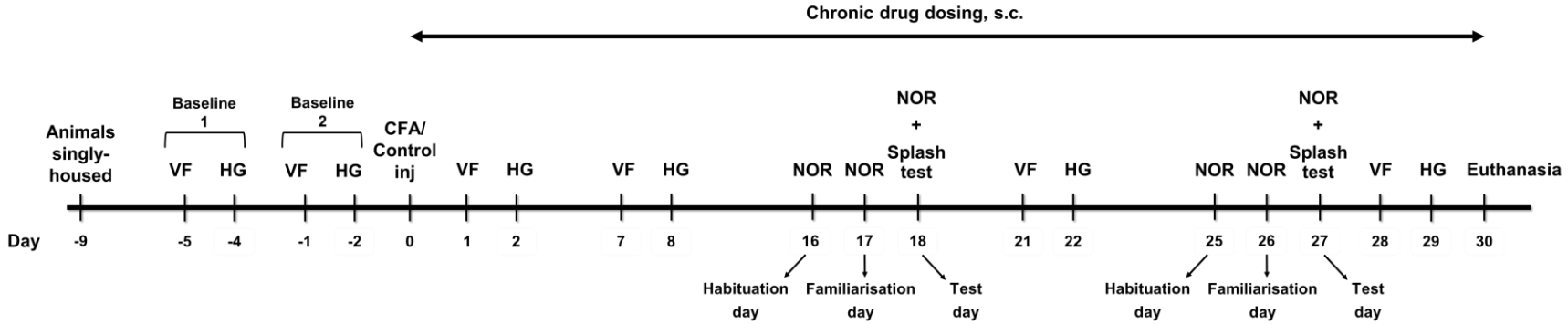


Figure 6.1: Schematic of the study outlining the timeline of behavioural tests. CFA: complete Freund's adjuvant, HG: Hargreaves' test, inj: injection, NOR: novel object recognition test, s.c.: subcutaneous, VF: von Frey test.

6.2.5.2. Novel object recognition test for the assessment of recognition memory

The novel object recognition (NOR) test was carried out as described previously in Chapter 5 (section 5.2.4.4.2). Briefly, the test was conducted in a circular open field arena over 3 consecutive days: habituation (exploration of the empty arena), familiarisation day (3x5 min trials for exploration of identical objects with 5 min inter-trial interval), and 24 h later test day (1x5 min trial for exploration of familiar and novel objects). In this study, NOR test was performed at two post-CFA time points, Day 16-18 and Day 25-27. Distinct sets of objects (identical and novel) were used for each test. In the first NOR task, the objects used were as before (Chapter 5): 500 mL plastic Coca-Cola® bottles (identical objects) and a plastic toy block structure (novel object). The objects used in the second NOR test were 473 mL metallic black Monster drink cans (identical objects) and a plastic rectangular grey bottle of approximately similar dimension (novel object). In all cases, the objects had no apparent natural significance to the rats.

6.2.5.3. Splash test for the assessment of motivation-related behaviour

The splash test was used to evaluate self-grooming behaviour, which is an ethologically relevant behaviour in rodents (Kalueff et al., 2016). Grooming is cleaning the fur of the animal by licking or scratching. A decrease in self-grooming in rodents can used be a marker of anhedonia (motivation-related behaviour) (Ducottet and Belzung, 2004; Yalcin et al., 2005; Hu et al., 2017). Immediately after the NOR test on Day 18 and 27, the animals were assessed in the splash test according to protocols described previously (Ducottet and Belzung, 2004; Yalcin et al., 2005; Rosa et al., 2014; Hu et al., 2017). After the 5-min NOR test day trial, the animal was transferred to another behavioural testing room and placed in a clear Plexiglass box (30x30x40 cm^3, 30 lux) for 10 min. After the initial habituation to the arena, 10% sucrose solution was squirted twice on the dorsal coat of the rat using a spray bottle approximately 10 cm away from the body of the animal. The animal was immediately returned to the arena and observed for another 10 min. Because of its viscosity, the sucrose solution dirties the coat/fur and initiates grooming. Moreover, the palatability of the sucrose solution permits the persistence of the grooming behaviour.

A video camera located underneath the arena recorded the test session. Grooming behaviour (bouts consisting of nose/face stroking, head washing, and body licking including the dorsum) was manually scored later using EthoVision software (EthoVision

XT, version 11.5; Noldus, Wageningen, Netherlands). The total duration of grooming during the 10-min test session and latency to start grooming are used as indices of self-care and motivational behaviour. The arena was cleaned with warm soapy solution and between animals.

6.2.6. Statistical analysis

The SPSS statistical software (IBM SPSS Statistics, version 24 for Windows; SPSS Inc, Chicago, IL, USA) was used to analyse all data. In all datasets the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was considered an outlier and excluded from subsequent analysis. The normality and homogeneity of variance of the datasets were checked using Shapiro-Wilk and Levene's tests, respectively. The timecourse behavioural data were analysed with repeated measures analysis of variance (RM ANOVA) with CFA injection and drug treatment as between-subject factors and time/day as within-subject factor. For RM ANOVA, sphericity of the datasets was checked with Mauchly's Test for Sphericity; if the assumption of sphericity was violated, a Greenhouse-Geisser correction was used. Other behavioural data were analysed using two-way ANOVA with CFA injection and drug treatment as factors. Preference index in NOR test was analysed using three-way ANOVA with day (familiarisation/test) as an additional factor. *Post hoc* Student-Newman-Keuls (SNK) test was carried out for pairwise group comparisons, where appropriate. The level of significance was deemed $p<0.05$. If the data were not normally distributed and/or the variance was not homogeneous, three transformations were applied, in the order: square root, natural logarithm, and ranking of the data values to evaluate if parametric statistics can be used. In addition, if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular dataset being analysed, parametric statistical approach was still employed (Moore et al., 2009).

If any dataset was ordinal (e.g. VF test data) or found to be not normally distributed and/or the variance was not homogeneous even after transformation, non-parametric statistical analysis was performed using Kruskal-Wallis (KW) one-way analysis of variance by rank, followed by *post hoc* Mann-Whitney U-test (*p*-value was adjusted using Bonferroni-Holm correction for multiple comparisons), where appropriate. Friedman's test followed by Wilcoxon signed-rank test was employed to assess timecourse data of VF test. In all cases statistical analysis of a dataset was carried out for all the groups together. However,

in some instances, the groups are graphed separately to present the results clearly in a reader-friendly format. Data are expressed as either mean ± standard error of the mean (S.E.M) or median with interquartile range (IQR) depending on the statistical approach undertaken, parametric or non-parametric, respectively.

6.3. Results

6.3.1. Effects of SNC80 and DIPPA, alone or in combination, on CFA-induced mechanical allodynia

Friedman's test revealed a significant difference in paw withdrawal thresholds among experimental groups for the ipsilateral ($\chi^2(5)=86.667$, ***p*<0.001**), but not for the contralateral ($\chi^2(5)=8.483$, $p>0.05$), paw. *Post hoc* Wilcoxon test showed that the ipsilateral paw withdrawal threshold at baseline 1 was significantly higher than baseline 2 (***p*<0.001**). Hence, subsequent comparisons of paw withdrawal thresholds between pre- and post-CFA time points were performed relative to baseline 2. *Post hoc* Wilcoxon test also showed that the hindpaw withdrawal threshold on baseline 2 was significantly higher than Days 1, 7, 21, and 28 on the ipsilateral side in CFA-injected rats (all four post-CFA time points vs baseline 2, ***p*<0.01**), indicating CFA-induced mechanical allodynia. Wilcoxon test indicated there were no significant differences in ipsilateral hindpaw withdrawal threshold between baseline 2 and post-CFA time points in the non-CFA-injected (control) rats (all four post-CFA time points vs baseline 2, $p>0.05$).

On the contralateral side (Figure 6.2A), KW test revealed that there was no significant main effect on paw withdrawal thresholds at baseline or at post-CFA injection time points [baseline 2: $\chi^2(7)=6.957$, $p>0.05$; Day 1: $\chi^2(7)=9.277$, $p>0.05$; Day 7: $\chi^2(7)=8.346$, $p>0.05$; Day 21: $\chi^2(7)=10.137$, $p>0.05$; Day 28: $\chi^2(7)=11.413$, $p>0.05$].

On the ipsilateral side (Figure 6.2B), KW test showed that there was no significant main effect on paw withdrawal threshold at baseline ($\chi^2(7)=3.430$, $p>0.05$). KW analysis showed significant main effects on paw withdrawal thresholds on Day 1, 7, 21, and 28 post-CFA injection [Day 1: $\chi^2(7)=33.213$, ***p*<0.001**; Day 7: $\chi^2(7)=56.355$, ***p*<0.001**; Day 21: $\chi^2(7)=58.817$, ***p*<0.001**; Day 28: $\chi^2(7)=49.523$, ***p*<0.001**]. Further *post hoc* tests at each post-CFA time point revealed that, regardless of drug/vehicle treatment, CFA-injected rats exhibited significantly lower paw withdrawal thresholds in comparison to respective control counterparts (Day 1: CFA vs non-CFA groups, ***p*<0.05**, Day 7: CFA vs non-CFA groups, ***p*<0.001**, Day 21: CFA vs non-CFA groups, ***p*<0.001**, Day 28: CFA vs non-CFA groups, ***p*<0.001**), further indicating development of CFA-induced mechanical allodynia in the ipsilateral paw. In the control rats, chronic administration of DIPPA significantly reduced paw withdrawal threshold to mechanical stimulation on Day 7, compared to

vehicle-treated counterpart (Day 7: non-CFA-DIPPA vs non-CFA-vehicle, ***p*<0.05**); this effect of DIPPA was not observed in other post-CFA time points.

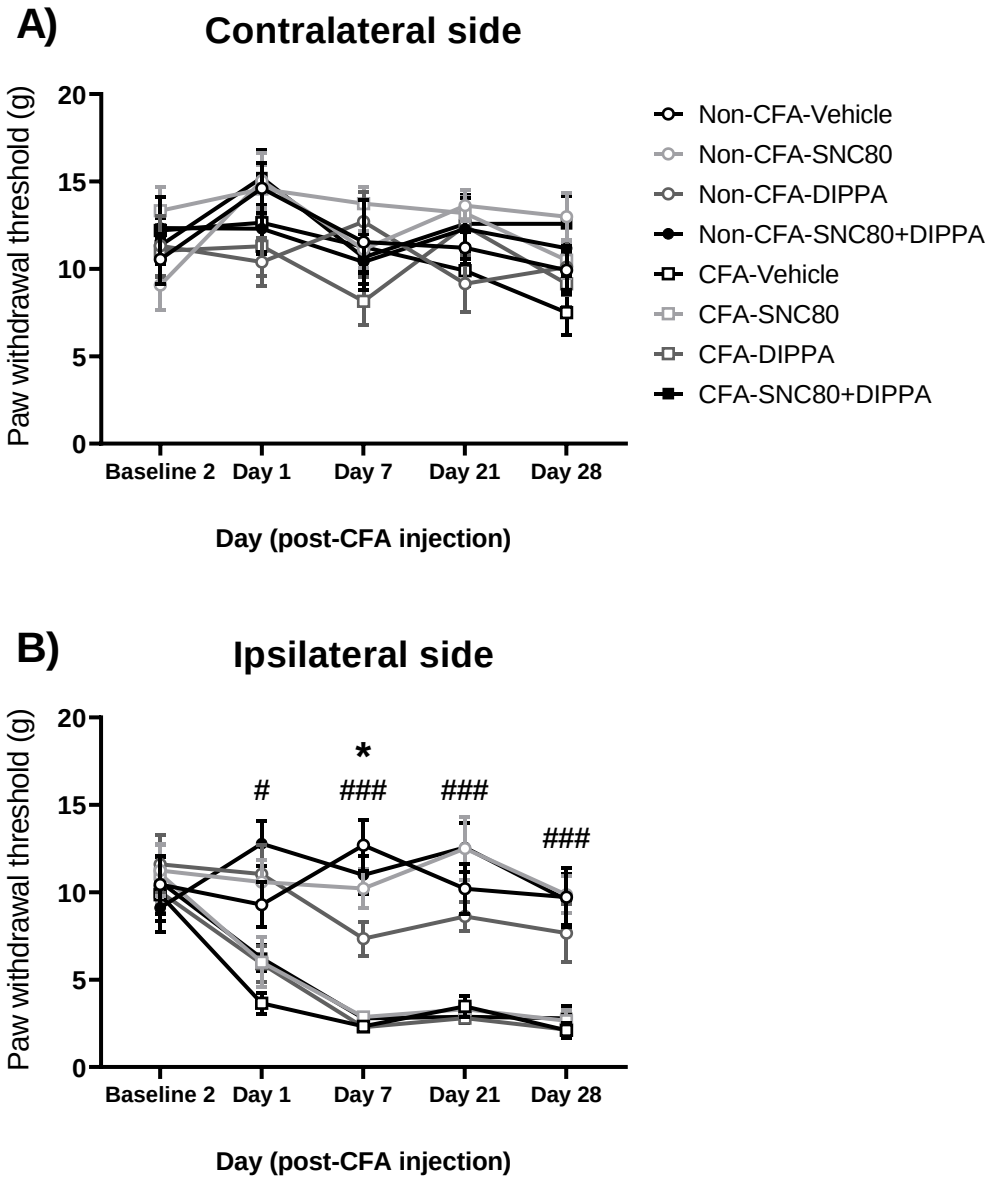


Figure 6.2: Timecourse of the effects of SNC80 and DIPPA, alone or in combination, on the mechanical withdrawal threshold of the (A) contralateral and (B) ipsilateral hindpaw in the control and CFA-injected SD rats using VF test. Data are expressed as median with IQR, n=8-10/group. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ (CFA vs non-CFA counterpart); $^{*}p<0.05$ (non-CFA-DIPPA vs non-CFA-vehicle). CFA: complete Freund's adjuvant, SD: Sprague-Dawley, VF: von Frey.

6.3.2. Effects of SNC80 and DIPPA, alone or in combination, on CFA-induced hypersensitivity to noxious heat stimulus

On the contralateral side (Figure 6.3A), RM ANOVA showed significant effects of time ($F_{4,256}$=2.698, ***p*<0.05**) in tests of within-subjects effects on response latency to noxious heat stimulus. Further *post hoc* analysis revealed that there was no significant difference between relevant experimental groups.

RM ANOVA revealed significant effects of time ($F_{3.228,206.576}$=51.790, ***p*<0.001**) and time x CFA ($F_{3.228,206.576}$=44.626, ***p*<0.001**) in tests of within-subjects effects on response latency to noxious heat stimulus on the ipsilateral side. A significant overall effect of CFA ($F_{1,64}$=505.801, ***p*<0.001**) was also observed in tests of between-subjects effects on response latency. *Post hoc* SNK test showed that response latency between the experimental groups was not different at baseline. Furthermore, CFA-injected rats exhibited significantly lower response latency, compared to non-CFA-injected (control) counterparts, on Day 2, 8, 22, and 29 (all post-CFA time points: CFA vs non-CFA groups, ***p*<0.05**), indicating development of CFA-induced heat hypersensitivity in the ipsilateral paw (Figure 6.3B). Chronic administration of SNC80 and DIPPA, alone or in combination, did not affect response latency to radiant heat in either control or CFA-injected rats at any time point.

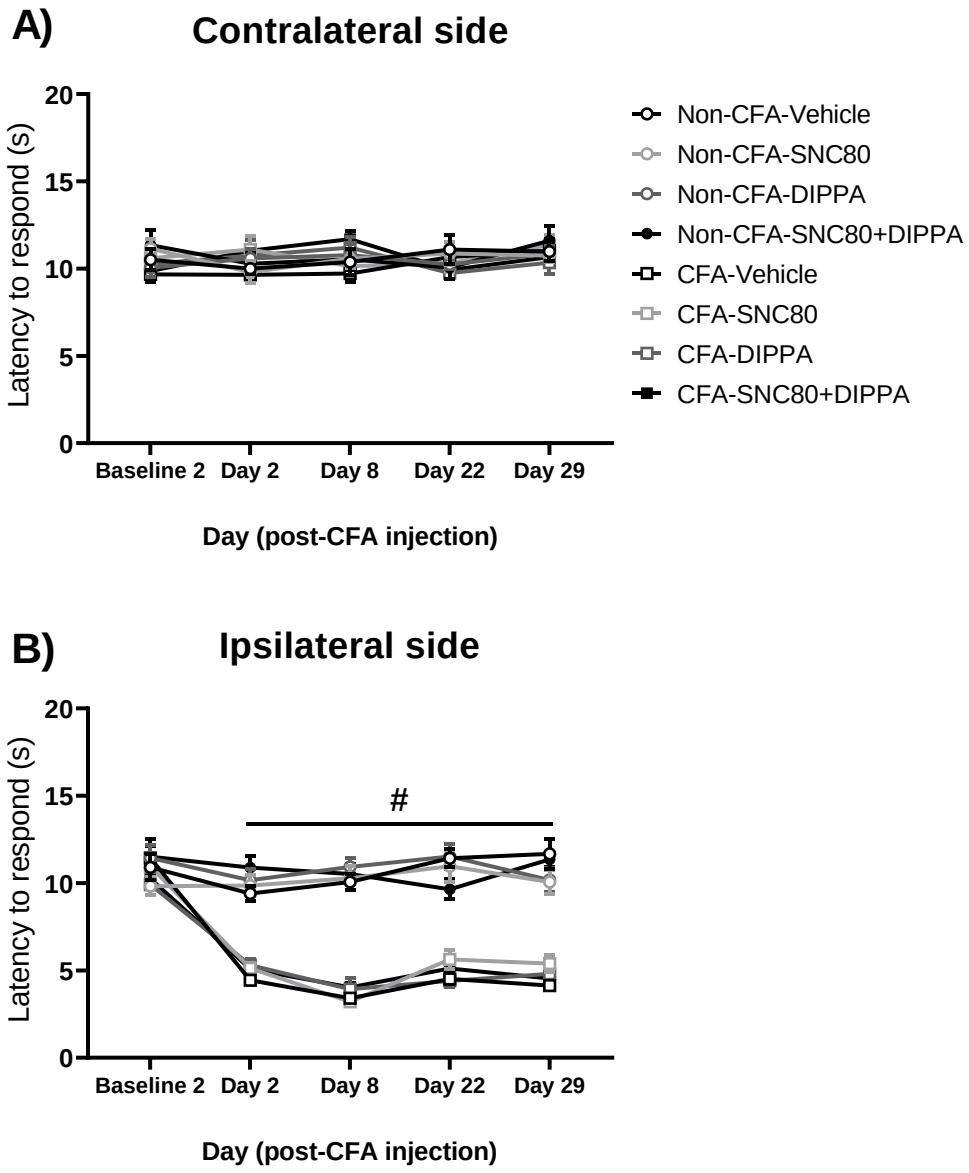


Figure 6.3: Timecourse of the effects of SNC80 and DIPPA, alone or in combination, on the withdrawal latency to noxious heat stimulus of the (A) contralateral and (B) ipsilateral hindpaw in the control and CFA-injected SD rats using HG test. Data are expressed as mean ± SEM, n=8-10/group. $^{\#}p<0.05$ (CFA vs non-CFA counterpart). CFA: complete Freund's adjuvant, HG: Hargreaves' test, SD: Sprague-Dawley.

6.3.3. Effects of SNC80 and DIPPA, alone or in combination, on recognition memory

The NOR test was employed to assess long-term recognition memory at two post-CFA time points, Day 18 and Day 27. On Day 18 post-CFA injection (Figure 6.4), two-way ANOVA revealed significant effect of drug treatment ($F_{3,72}$=5.679, ***p*<0.01**) only, but not of CFA ($F_{1,72}$=0.179, p>0.05) or CFA x treatment interaction ($F_{3,72}$=1.416, p>0.05), on time spent exploring the novel object. Further *post hoc* SNK test revealed that there were no significant differences between relevant groups (Figure 6.4A). Of note, a trend for DIPPA-induced reduction in novel object exploration was observed in the CFA-injected rats (CFA-DIPPA vs CFA-vehicle, p=0.077), which failed to reach statistical significance. There were no significant effects of CFA, treatment, and CFA x treatment interaction on time spent exploring the familiar objects in the arena (Figure 6.4B).

Furthermore, three-way ANOVA revealed significant effects of day ($F_{1,145}$=393.716, ***p*<0.001**) and drug treatment ($F_{3,145}$=3.965, ***p*<0.05**) in the measure of preference index for the novel object on test day relative to the object in that location on familiarisation day (Figure 6.4C). *Post hoc* SNK analysis showed that all experimental groups spent more time exploring the novel object on test day in comparison to object 3 in the same location on familiarisation day (***p*<0.05**). In addition, two-way ANOVA found significant effects of treatment ($F_{3,72}$=5.679, ***p*<0.01**) only on discrimination index which compares the time spent exploring the novel object relative to the familiar objects in the arena on the test day. However, *post hoc* test showed there was no significant difference between relevant groups (Figure 6.4D). Again, chronic DIPPA administration produced a strong trend in reducing discrimination index for the novel object in CFA-injected rats (CFA-DIPPA vs CFA-vehicle, p=0.063), which failed to reach statistical significance.

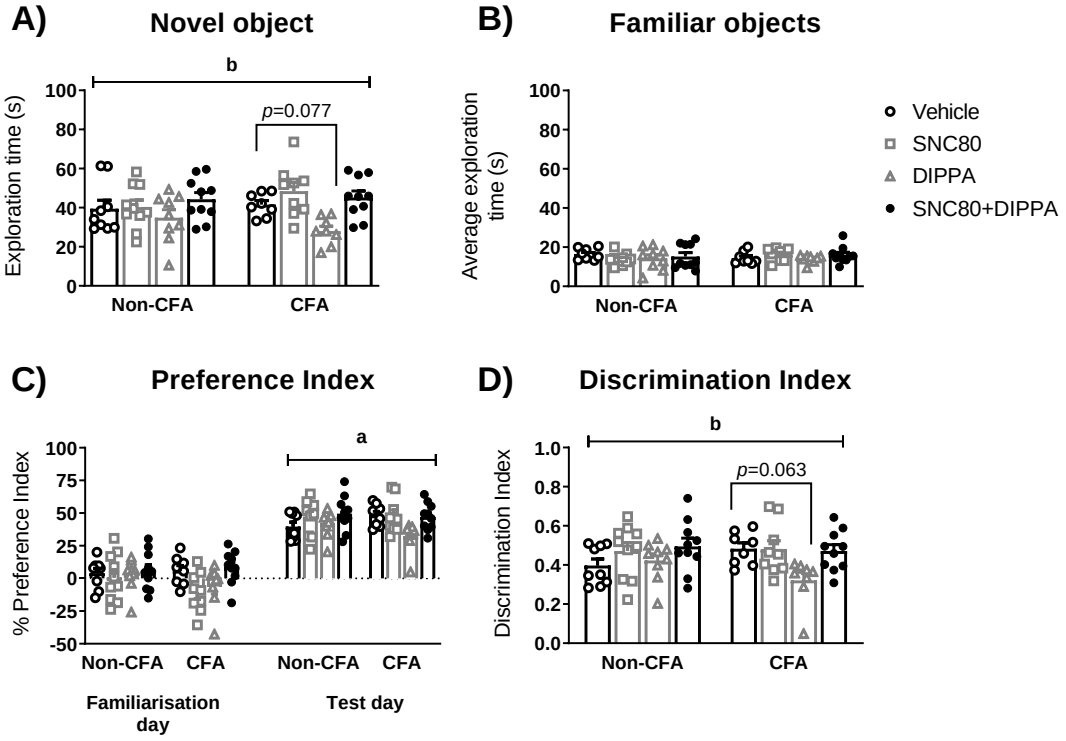


Figure 6.4: Effects of SNC80 and DIPPA, alone or in combination, on recognition memory in control or CFA-injected SD rats on Day 18 post-injection. (A) Time spent exploring the novel object on the test day in non-CFA vs CFA rats. (B) Average time spent exploring the familiar objects on the test day in non-CFA vs CFA rats. (C) Percentage preference index comparing exploration time with the novel object on test day relative to object 3 on familiarisation day in non-CFA vs CFA rats. (D) Discrimination index comparing exploration time with the novel object relative to the familiar objects on the test day in non-CFA vs CFA rats. Data are expressed as mean ± SEM, n = 8-10/group. a: effect of day (p<0.001), b: effect of drug treatment: (p<0.01). CFA: complete Freund's adjuvant, SD: Sprague-Dawley.

With regards to the NOR test on Day 27 post-CFA injection (Figure 6.5), two-way ANOVA indicated that there were no significant effects of the factors on time spent exploring the novel object or familiar objects or on discrimination index [Novel object- CFA: $F_{1,76}$=1.820, p>0.05; treatment: $F_{3,76}$=0.585, p>0.05; CFA x treatment interaction: $F_{3,76}$=0.594, p>0.05. Familiar objects- CFA: $F_{1,76}$=0.339, p>0.05; treatment: $F_{3,76}$=0.049,

p>0.05; CFA x treatment interaction: $F_{3,76}$=0.348, *p*>0.05. Discrimination index: CFA: $F_{1,76}$=0.109, *p*>0.05; treatment: $F_{3,76}$=0.516, *p*>0.05; CFA x treatment interaction: $F_{3,76}$=0.119, *p*>0.05. Figure 6.5A, 6.5B, and 6.5D]. Three-way ANOVA revealed a significant effect of day ($F_{1,153}$=366.258, ***p*<0.001**) only on preference index. Further *post hoc* SNK test showed that all experimental groups spent more time exploring the novel object on test day in comparison to object 3 in the same location on familiarisation day (***p*<0.05**) (Figure 6.5C).

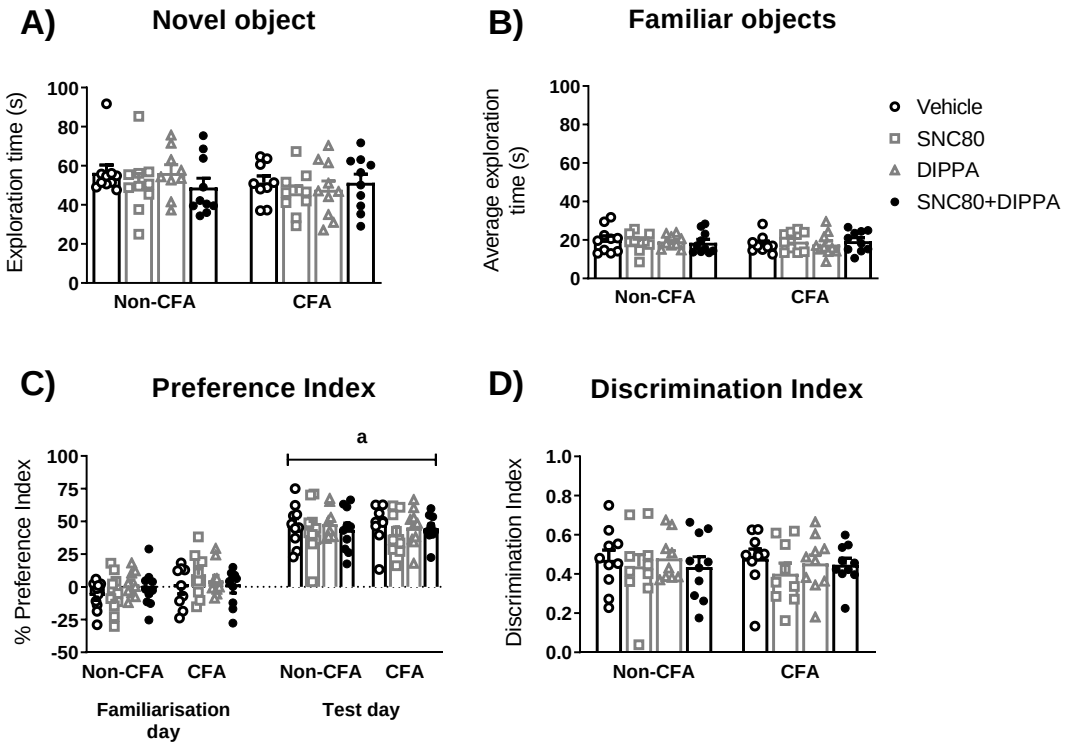


Figure 6.5: Effects of SNC80 and DIPPA, alone or in combination, on recognition memory in control or CFA-injected SD rats on Day 27 post-injection. (A) Time spent exploring the novel object on the test day in non-CFA vs CFA rats. (B) Average time spent exploring the familiar objects on the test day in non-CFA vs CFA rats. (C) Percentage preference index comparing exploration time with the novel object on test day relative to object 3 on familiarisation day in non-CFA vs CFA rats. (D) Discrimination index comparing exploration time with the novel object relative to the familiar objects on the test day in non-CFA vs CFA rats. Data are expressed as mean ± SEM, n = 8-10/group. a: effect of day (*p*<0.001). CFA: complete Freund's adjuvant, SD: Sprague-Dawley.

6.3.4. Effects of SNC80 and DIPPA, alone or in combination, on motivation-related behaviour

Sucrose splash test was used to evaluate ethologically relevant anhedonic-like behaviour in the rats at two post-CFA time points, Day 18 and Day 27 (Figure 6.6 and 6.7, respectively). On Day 18 post-CFA injection, KW test revealed a significant main effect ($\chi^2(7)=14.944$, $\boldsymbol{p<0.05}$) on time spent grooming but further *post hoc* test indicated that there was no significant difference between relevant experimental groups (Figure 6.6A). Moreover, KW test showed no significant main effect ($\chi^2(7)=4.165$, $p>0.05$) on latency to start grooming in the splash test (Figure 6.6B).

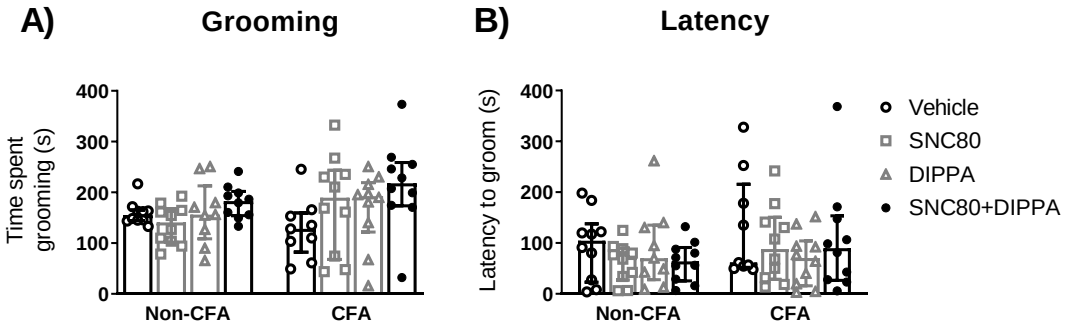


Figure 6.6: Effects of SNC80 and DIPPA, alone or in combination, on grooming activity in the splash test in control or CFA-injected SD rats on Day 18 post-injection. (A) Time spent grooming during 10-min splash test. (B) Latency to start grooming after spraying 10% sucrose solution on the dorsal coat. Data are expressed as median with IQR, n = 9-10/group. CFA: complete Freund's adjuvant, SD: Sprague-Dawley.

On Day 27 post-CFA injection, KW analysis revealed that there were no significant main effects on grooming time ($\chi^2(7)=7.735$, $p>0.05$; Figure 6.7A) or latency ($\chi^2(7)=1.716$, $p>0.05$; Figure 6.7B) to groom in the splash test.

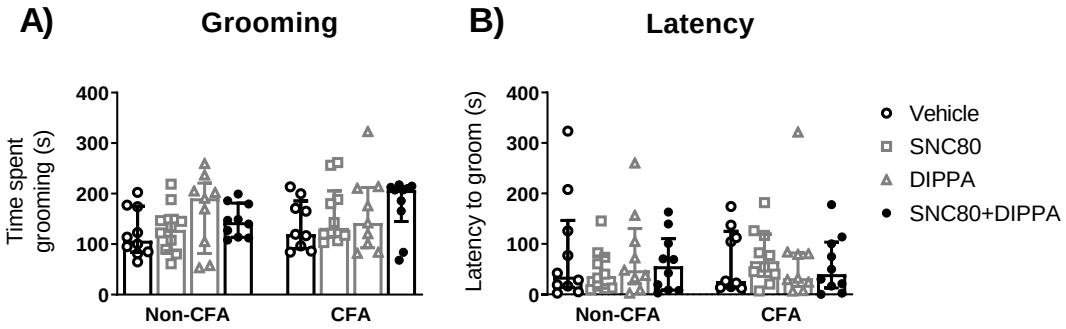


Figure 6.7: Effects of SNC80 and DIPPA, alone or in combination, on grooming activity in the splash test in control or CFA-injected SD rats on Day 27 post-injection. (A) Time spent grooming during 10-min splash test. (B) Latency to start grooming after spraying 10% sucrose solution on the dorsal coat. Data are expressed as median with IQR, n = 9-10/group. CFA: complete Freund's adjuvant, SD: Sprague-Dawley.

6.4. Discussion

In the present study we observed that chronic administration of SNC80 or DIPPA did not affect mechanical allodynia or heat hyperalgesia induced by CFA injection in SD rats. CFA injection did not induce any deficit in recognition memory (NOR test) or motivation-related behaviour (splash test) at the time points tested post-CFA injection (Day 18 and Day 27). Chronic administration of DIPPA tended to impair performance in the NOR test on Day 18 in the CFA-injected, but not in the control, rats. There was no effect of SNC80 or DIPPA on grooming behaviour in the splash tests. Furthermore, chronic administration of SNC80 and DIPPA together did not affect any of the behavioural domains (nociceptive responding, depressive-like, and cognition) in either control or CFA-injected rats.

Intraplantar injection of CFA into the hindpaw induced mechanical allodynia and thermal hyperalgesia in the rats, as expected. Chronic administration of SNC80 did not affect nociceptive responding in control or CFA-injected rats. Previous studies have shown acute systemic or intracerebral administration of SNC80 effectively reduced CFA-induced mechanical and thermal hypersensitivity in rats and mice (Fraser et al., 2000; Cao et al., 2001; Gavériaux-Ruff et al., 2008, 2011; Pradhan et al., 2013). Only two studies to date have investigated the effects of repeated or chronic administration of SNC80 in mice in pain models. Repeated administration of SNC80 (10 mg/kg) in CFA-injected mice over 5 days produced significant antinociception in the beginning of the schedule but also resulted in analgesic tolerance (Pradhan et al., 2010). Moye and coworkers have recently reported that chronic daily treatment with SNC80 (10 mg/kg) for 11 days reduced peripheral and cephalic allodynia in a mouse model of chronic migraine, although the authors observed limited development of opioid-induced hyperalgesia following chronic SNC80 treatment (Moye et al., 2019). Thus, it appears that the low dose (1 mg/kg) of SNC80 used in our study may account for the lack of effect on nociceptive responding in VF and HG tests in the rats both at an acute time point (Day 1) and following chronic administration. No study to date has investigated the effects of chronic administration of DIPPA in tests of pain. We demonstrated earlier in Chapter 4 that acute DIPPA (2.5 mg/kg) injection reduced formalin-evoked nociceptive behaviour. Like SNC80, the low dose (1 mg/kg) of DIPPA in general did not affect nociceptive responses in the present study. Interestingly, repeated DIPPA treatment transiently reduced paw withdrawal threshold in the control, but not in the CFA-injected, rats on Day

7. However, this apparent pronociceptive effect of DIPPA might be due to the modest increment observed in mechanical threshold in the vehicle group at that time point.

The present study examined CFA-induced cognitive deficit on Day 18 and 27 following intraplantar CFA injection into the hindpaw. Vehicle-treated CFA-injected rats did not exhibit impairment in object recognition memory in the NOR test at either post-CFA time point, compared to control counterparts. The results observed for Day 18 in this study are at odds with the previous study discussed in Chapter 5, where CFA-injected SD rats displayed a modest reduction in novel object exploration time compared to control counterparts. Methodological differences (e.g. repeated injection procedure, different set of testing procedures) between the two studies may account for the discrepant results. Intraplantar CFA injection during neonatal period (postnatal day 1) impairs long-term recognition memory in the NOR task performed during adulthood (postnatal day 60) in rats (Amaral et al., 2015), suggesting that chronic pain during early life may induce cognitive deficits in adulthood. Hence, future studies may investigate any deficit in recognition memory in the CFA model at later time points than in our study.

Chronic SNC80 administration did not affect recognition memory in control or CFA-injected rats in the current study. A recent study has shown that 3CS-nalmefene, a mixed MOP antagonist/partial DOP agonist, prevents impairment in object displacement learning in rats in the interferon-alpha model of depression (Callaghan et al., 2018), suggesting DOP may have a role in learning and memory. However, the dose of SNC80 employed in our study might be too low to elicit any effect in the NOR task. On the other hand, we observed strong trends for DIPPA-induced impairment in recognition memory in the CFA-treated rats, but not in the non-CFA (control) counterparts, suggesting that the pain status of the animal may alter the effect of drug on memory performance (Baiamonte et al., 2013). In our chronic dosing schedule, we maintained the 24 h administration time point of DIPPA prior to any behavioural testing, as published previously (Chang et al., 1994b; Carr and Lucki, 2010; Carr et al., 2010) to avoid KOP agonist-like activity of DIPPA within the first 4 h of administration (Chang et al., 1994b). However, previous reports have shown that acute administration of KOP antagonists prevent KOP agonist- or stress-induced impairment in recognition memory (NOR and water maze tasks) in rodents (Daumas et al., 2007; Carey et al., 2009; Paris et al., 2011). The exact pharmacodynamic profile of DIPPA with regards to its intrinsic activity at the KOP is not yet fully known and *in vivo* studies have suggested mixed agonist/antagonist

results in different behavioural paradigms (Burton and Gebhart, 1998; Kuzmin et al., 2000; Terner et al., 2005; Carr and Lucki, 2010; Carr et al., 2010). Therefore, further studies are needed to elucidate the mechanisms underlying differential effects of KOP modulation on cognition-related behaviours in the CFA model of chronic inflammatory pain.

We also investigated chronic inflammatory pain-induced anhedonic (motivation-related) behaviour on Day 18 and 27 post-CFA injection. Anhedonia (defined as loss of interest or pleasure) is one of the core symptoms of depression (Uher et al., 2014). We assessed anhedonia by measuring self-grooming in the rats in the splash test. Self-grooming is a complex innate homeostatic behaviour in rodents that is highly regulated by specific brain regions including the cortex, striatum, and amygdala (Kalueff et al., 2016). Previous studies have shown that grooming is reduced in rodents in the splash test following different kinds of behavioural (Yalcin et al., 2008; Amiri et al., 2016; Sadeghi et al., 2016; Hu et al., 2017) and biological (Sens et al., 2017) stressors and has been interpreted as a marker of anhedonia. Treatment with typical antidepressants prevent stress-induced grooming deficit in rodents (Yalcin et al., 2008; Sadeghi et al., 2016). The present study did not find any CFA-induced deficit in grooming behaviour at both testing time points. A few previous studies have reported CFA-induced anhedonic behaviour in the sucrose preference test in rodents (Shi et al., 2010; Refsgaard et al., 2016; Zhang et al., 2016). The difference in testing paradigm used between our study and those published may account for the discrepant results.

With regard to treatment, no drug effect on grooming was observed in the control or CFA-injected rats at either time points in the present study. Antidepressant properties of SNC80 have been found in rodents in a number of behavioural paradigms, such as the forced swim (Broom et al., 2002; Jutkiewicz et al., 2004; Saitoh et al., 2004; Nozaki et al., 2014), tail suspension (Henry et al., 2018), and social defeat (Henry et al., 2018). Furthermore, repeated daily dosing with SNC80 (3 and 10 mg/kg) for 8 days attenuates anxiety-like behaviour in the EPM in olfactory bulbectomized rats (Saitoh et al., 2008). As for KOP antagonists such as nor-BNI and JDTic, they have been shown to produce antidepressant-like effect in classical rodent models of depression (Mague et al., 2003; Shirayama et al., 2004; Beardsley et al., 2005; Grimwood et al., 2011). A recent study has also demonstrated that relatively short-acting KOP antagonists (LY2444296 and LY2795050) reduce grooming deficits induced by the KOP agonist salvinorin-A in the splash test

(Butelman et al., 2019). These studies highlight the potential of DOP agonists and KOP antagonists in depression-like states. Hence, further investigation is warranted to explore manifestation of depressive-like behaviours in chronic inflammatory pain and any effect of opioid modulation thereon.

Our study is the first to investigate the effects of chronic administration of SNC80 and DIPPA together in the CFA model of chronic inflammatory pain. Synergistic antidepressant-like effects of combining a DOP agonist (ADL5859) and a KOP antagonist (LY2444296) have been reported recently (Huang et al., 2016). The authors demonstrated that each drug on its own significantly reduces immobility in the forced swim test in mice. However, in combination, much lower doses of ADL5859 and LY2444296 were needed to produce comparable antidepressant-like effects, devoid of any locomotor activation effect (Huang et al., 2016). Hence, given that a DOP agonist and a KOP antagonist have distinct pharmacological profiles, such a synergism may allow the use of both drugs at lower doses to achieve desired behavioural output with fewer side effects. Although in the present study combination of SNC80 and DIPPA did not produce any significant behavioural effect in the rats, further investigation is warranted to explore the potential usefulness of this combination strategy.

In conclusion, these results did not provide any evidence for CFA-induced anhedonia or impairment of recognition memory in SD rats at the time points tested. Overall, chronic administration of SNC80 and DIPPA, alone or in combination, did not affect CFA-induced mechanical allodynia or heat hyperalgesia at the doses tested. Moreover, trends were seen for DIPPA-induced impaired recognition memory in CFA-injected rats only at a discrete time point. The functional relevance of such observation warrants further investigation. Lastly, chronic modulation of DOP and/or KOP appear not to affect grooming behaviour in the sucrose splash test at the doses tested.

7. Chapter 7: General Discussion

Affective disorders (anxiety and depression) and pain are among the most debilitating conditions worldwide. A substantial body of research indicates that pain and negative affect share a complex reciprocal relationship, whereby the presence of one condition can modulate the other (Wiech and Tracey, 2009; Hawker et al., 2011; Jennings et al., 2014). However, the neurobiological mechanisms underlying this association are poorly understood. Several studies have shown that anxiety and depressive disorders significantly increase the risk of developing chronic headache and musculoskeletal (including low back and neck/shoulder) pain (Ohayon and Schatzberg, 2003; Grover et al., 2012; Peres et al., 2017). Pain and negative affective states frequently coexist (Poole et al., 2009; Gadermann et al., 2012), which further aggravates the severity of each individual disorder, reducing the effectiveness of pharmacological treatment. Such comorbid conditions significantly impair the quality of life of the patients and impose a huge socioeconomic burden. As such, understanding the neurobiology of this interaction may facilitate the development of improved therapeutics for the management of pain, affective disorders, and their comorbidity. In this regard, it is imperative to investigate the participation of the opioid system in the interaction between pain and negative affect, given the key role played by this system in modulating both pain and affect/emotion.

The aim of the work described in this thesis was largely to examine nociceptive responding to thermal (acute) and inflammatory (persistent or chronic) stimuli in the WKY rat, a genetic model of negative affect. The present work also aimed to determine if the behavioural changes were related to alterations in the opioid system, particularly the MOP and KOP system, in key brain regions implicated in pain and affect. In addition, the effects of pharmacological manipulation of MOP and KOP were investigated in WKY and SD rats on acute heat and persistent inflammatory pain and anxiety-/depression-related behaviours. Moreover, the effects of pharmacologically modulating MOP in the ventrolateral subcolumn of PAG on inflammatory pain and the resulting influence on the descending inhibitory pain pathway were compared between WKY and SD rats. Additionally, the effects of CFA-induced chronic inflammation on nociceptive responding and chronic pain-associated negative affect and cognitive impairment were examined in WKY and SD rats. Lastly, we also investigated the effects of pharmacologically modulating KOP and DOP on nociceptive responding, depressive-, and cognition-related behaviour in the rat CFA model.

The main findings reported and discussed in this thesis include:

1) WKY rats were less sensitive to the antinociceptive effects of systemically administered MOP agonist morphine in tests of acute thermal (hot plate test) and persistent inflammatory (formalin test) pain, compared to SD rats.
2) WKY rats displayed reduced responsivity to the antinociceptive effects of morphine administered directly into the vlPAG, compared to SD counterparts, in the formalin model of persistent inflammatory pain. Furthermore, concomitant changes in the expression of c-Fos in downstream neural substrates (NRM and lumbar spinal dorsal horn) were blunted in the WKY versus SD rats.
3) WKY rats were hyporesponsive to pharmacological modulation of KOP in the formalin model of persistent inflammatory pain.
4) There were receptor/prepropeptide-specific and region-dependent differences in the expression of opioid-related genes, particularly those within the MOP and KOP system, in key regions involved in processing pain and affect between WKY and SD rats (Table 7.1).
5) Mechanical allodynia was exacerbated in the WKY rats following intraplantar CFA injection into the hindpaw. Strain differences in the measure of social behaviour and spatial and recognition memory were apparent between WKY and SD rats. Additionally, recognition memory was impaired following CFA injection in the SD rats.
6) Chronic systemic administration of KOP antagonist and DOP agonist, alone or in combination, had no effects on CFA-induced mechanical allodynia or heat hyperalgesia at the doses tested in SD rats.

Chapters 2 and 3 examined the effects of pharmacological manipulation of the MOP via systemic administration and direct injection into the vlPAG, respectively, on nociceptive responding using two models of pain, acute heat and persistent inflammatory pain. The results from Chapter 2 showed that the systemic administration of the MOP agonist morphine produced antinociception in the hot plate and formalin tests in SD rats, effects attenuated by the MOP selective antagonist cyprodime. In both tests of nociception used here, WKY rats appeared to be less sensitive to morphine than SD rats, particularly to relatively low doses of morphine. Reduced responsivity to opioids in WKY rats, compared with SD rats, has been reported previously in the hot plate and warm water tail withdrawal tests (Sitsen et al., 1987; Hoffmann et al., 1998; Terner et al., 2003; Hestehave

et al., 2019b). Our results extend this finding to the formalin model of persistent inflammatory pain. These strain-related differences in morphine potency in nociceptive assays seem unlikely to be due to any effect of the drug on motor activity, as supported by the locomotor activity data from OF tests where 0.5 or 2 mg/kg morphine did not impair locomotor activity in either strain. Furthermore, equivalent plasma morphine levels in SD and WKY rats suggested that these differences in morphine sensitivity were unlikely to be due to differences in morphine pharmacokinetics in the two strains. Thus, considering the differential antinociceptive effects of morphine in WKY versus SD rats, it appears reasonable to suggest that there could be a lack of adequate engagement of the MOP system within descending pain circuitry in the WKY rats. Therefore, this hypothesis was further investigated in Chapter 3 with a focus on MOP in the vlPAG, an important region in the descending pain pathway for mediating MOP-induced antinociception (Sharpe et al., 1974; Yaksh et al., 1976b; Lane et al., 2005; Loyd et al., 2008; Morgan et al., 2009). The results showed that WKY rats were hyporesponsive to the antinociceptive effects of morphine injected into the vlPAG, compared to SD counterparts, in the persistent inflammatory (formalin) pain model. Although there are no studies to date specifically examining neural substrates within the descending pain pathway in relation to the opioid system in WKY rats, there is some evidence that the PAG is involved in hyperalgesia in this rat strain. Previous work in our laboratory has demonstrated subcolumn-specific alterations in the signalling of the transient receptor potential vanilloid 1 (TRPV1) (Madasu et al., 2016) and the peroxisome proliferator-activated (PPAR) receptors (Okine et al., 2017) in the PAG in WKY versus SD rats. Both of these systems have key roles in peripheral and central nociceptive processing (Caterina et al., 1997; McGaraughty et al., 2003; Russo et al., 2007; Costa et al., 2008; De Novellis et al., 2012). Though MOP expression in the vlPAG did not differ between WKY and SD rats, data presented in Chapter 3 identified the vlPAG as a key locus for the hyporesponsivity to MOP agonism in WKY rats in the formalin test. Future experiments could compare the activity of the MOP (e.g. using [^{35}S]GTPγS binding assay) in the vlPAG of WKY versus SD rats to assess any strain-related alteration in receptor functionality that will further support the pharmacological findings. Additionally, examining the components of the MOP-induced intracellular signalling cascade in the vlPAG may help to reveal neuromolecular underpinnings for hyperalgesia in the WKY rats. Also, it should be noted that the subcolumns of PAG have different but overlapping neuronal connections (Rizvi et al., 1991; An et al., 1998), playing distinct roles in stress and aversive responding (Keay

and Bandler, 2001). Therefore, further pharmacological investigation is necessary to unravel any relative contribution of the other PAG subcolumns (dlPAG and lPAG) to differential MOP-mediated response in the WKY rats.

Opioid-induced antinociception involves activation of the descending inhibitory pain pathway via GABAergic disinhibition (Lau and Vaughan, 2014). We found that intra-vlPAG injection of morphine in SD rats reduced formalin-evoked nociceptive behaviour and was associated with an increase in c-Fos expression in the NRM, one of the principal nuclei of the RVM involved in opioid-induced antinociception (Young et al., 1984; Urban and Smith, 1994). The increase in expression of c-Fos likely reflects an increase in RVM OFF cell activity (Fields et al., 1983b; McGaraughty et al., 1993; Tortorici and Morgan, 2002). Furthermore, there was a concomitant reduction in c-Fos expression in the lumbar segment of the DHSC, which likely reflects top-down inhibitory modulation of spinal nociceptive transmission (Presley et al., 1990; Gogas et al., 1991, 1996), reducing formalin-evoked nociception. These changes in c-Fos expression in NRM and DHSC were blunted in the WKY rats following intra-vlPAG morphine administration. The PAG-RVM-spinal cord circuit is a well-characterised component of the descending pain pathway (Millan, 2002). Thus, a reduced MOP-induced engagement of the descending inhibitory pain pathway from the vlPAG may underpin the hyperalgesic response to noxious inflammatory stimuli in the WKY rats, compared to SD counterparts. Electrophysiological studies would be useful here in understanding any strain-related functional differences in the activity of ON and OFF cells in the RVM. A limitation of my work here is that the identity of the neurons expressing c-Fos in the RVM and DHSC is not known at present. In this respect, future studies using double labelling immunohistochemistry technique will help to identify the c-Fos expressing neurons in the RVM and DHSC and could provide better mechanistic understanding regarding the deficit in MOP-induced signalling in the WKY rats.

While the PAG has been most studied in relation to hyperalgesic behaviour in the WKY rats, altered expression and/or functionality of the MOP within higher cortical and limbic brain regions of the WKY rats may also influence their hyporesponsivity to morphine, compared with SD rats. The CeA has dense reciprocal connections with the PAG, particularly with the lateral/ventrolateral subcolumns (Rizvi et al., 1991; Da Costa Gomez and Behbehani, 1995b; Neugebauer et al., 2004). Direct injection of morphine into the amygdala including the CeA produces antinociception in rats (Helmstetter et al., 1998;

Sabetkasaei et al., 2007; Rashvand et al., 2014), which can be antagonised by systemic administration of naloxone (Sabetkasaei et al., 2007). Moreover, administration of morphine into the amygdala increases the release of opioid peptides (enkephalins and β-endorphin) in the PAG (Ma and Han, 1991). The PAG also receives input from the mPFC (An et al., 1998; Gabbott et al., 2005). Electrical stimulation of the ACC attenuates the affective component of pain in the PEAP in a rat model of neuropathic pain, an effect that is blocked by lesioning the vlPAG without affecting the tactile sensitivity (LaBuda and Fuchs, 2005). Thus, higher brain regions can modulate neuronal activity at the level of PAG. We did not observe a global strain-related difference in the expression of the genes encoding MOP or POMC in regions associated with pain and affect, except for higher levels of MOP and POMC mRNA in the hippocampus and PFC, respectively, in naïve WKY versus SD rats (Table 7.1). Nevertheless, it will be interesting to further explore the effects of pharmacologically manipulating the MOP in specific subnuclei (such as the CeA and ACC) in the WKY rats to assess their involvement in hyperalgesic response. Moreover, techniques such as optogenetics could be employed to further unveil any differential input to the PAG (particularly to the ventrolateral subcolumn) from these higher brain sites, which may help to explain the characteristic behavioural phenotype of the WKY rats.

In Chapter 4, the effects of pharmacological manipulation of KOP via systemic administration of KOP-selective drugs, U50488 and DIPPA, were examined on nociceptive behaviour in WKY and SD rats. The prototypic KOP agonist U50488 produced antinociceptive effects in the SD rats in response to acute thermal (heat) and persistent inflammatory (formalin) stimuli, as was expected. One interesting finding of this study was that systemic administration of the KOP antagonist DIPPA alone reduced formalin-evoked nociceptive behaviour in the SD rats. Though the exact pharmacological profile of DIPPA at the KOP remains to be determined, studies have reported that DIPPA reduces anxiety- and depressive-like behaviours in rats (Carr and Lucki, 2010; Carr et al., 2010), which is in agreement with its KOP antagonist-like profile (Chang et al., 1994b, 1994a). Additionally, DIPPA (at doses >3mg/kg) attenuates U50488-induced antinociception in tests involving noxious acute heat stimulus (Kuzmin et al., 2000; Terner et al., 2005). Moon and colleagues have shown that intra-articular injection of 10 μM, but not 10 nM, of DIPPA reverses the antinociceptive effect of U50488 administered into the same site in a rat model of knee osteoarthritis (Moon et al., 2016). These findings

suggest that DIPPA has KOP antagonist-like effects *in vivo*. However, a complete interpretation of data from these published studies is difficult due to lack of inclusion of a treatment group that received only DIPPA, as in our study. The exception here is the report from Terner et al who has shown that DIPPA alone has mild antinociceptive effect in response to acute heat stimulus in rodents (Terner et al., 2005), which suggests agonist-like effects of DIPPA at the KOP. The findings discussed so far with respect to pharmacological effects of DIPPA also suggests another possibility that DIPPA may act as a partial agonist *in vivo*. Therefore, further *in vitro* and *in vivo* investigations are warranted to examine the intrinsic activity of DIPPA at the KOP.

Furthermore, in our study we observed that the WKY rats were hyporesponsive to the antinociceptive effects induced by U50488 or DIPPA in response to persistent inflammatory (formalin) stimulus, but not to heat (hot plate) stimulus. Such stimulus-dependent differences between the two strains with regard to efficacy of KOP modulators suggest that the KOP system is possibly hypofunctional in inflammatory pain in the WKY rats, but retains functionality in thermal pain in both strains. Noxious heat and chemical stimuli have different effects on the physiology of the somatosensory system. As mentioned earlier in Chapter 1, specialised transducer proteins such as the TRP channels on primary afferents (C- or Aδ-fibres) detect one or more stimulus modalities (Basbaum et al., 2009; Julius, 2013). Heat-sensitive afferents express TRPV1, an endogenous transducer of noxious heat and capsaicin (active ingredient of chili peppers) (Caterina et al., 1997, 2000). In addition to acute heat nociception, TRPV1 has a key role in mediating heat hypersensitivity induced by tissue injury and inflammation (Tominaga et al., 1998; Caterina et al., 2000; Davis et al., 2000), thus reflecting the ability of TRPV1 to act as a molecular integrator of thermal and chemical stimuli (Tominaga et al., 1998). Polymodal primary afferents also express transient receptor potential ankyrin 1 (TRPA1) receptor, which is involved in detecting chemical irritants that elicit inflammatory pain (Chen and Hackos, 2015). TRPA1 has been reported to be the principal mediator of nociceptive behaviour evoked by formalin *in vivo* (McNamara et al., 2007). Opioids, including those acting at KOP (Randic et al., 1995; Snyder et al., 2018), have been shown to effectively inhibit excitatory neurotransmission via nociceptive primary afferents, thereby producing antinociception (McCormack et al., 1998; Endres-Becker et al., 2007; Ikoma et al., 2007). Hence, there could be a differential modulation of the KOP on primary afferents that express TRPV1, TRPA1, or both in WKY versus SD rats, resulting in pain modality-

dependent differences in KOP antinociception in the two strains. Our findings here also underscore the importance of using multiple pain models when investigating genotype difference in opioid efficacy. Future studies examining the influence of both modality and strain will help in understanding whether genotype differences in opioid antinociception are limited to particular types of pain or not.

With regard to DIPPA, Carr and colleagues have examined DIPPA in tests related to negative affect and reported that whilst DIPPA is anxiolytic in both SD and WKY rats (Carr and Lucki, 2010), it has antidepressant-like effects in WKY rats, but not in SD counterparts (Carr et al., 2010). However, this strain-dependent effect of DIPPA was not seen in the FST in our study, possibly due to the low dose employed. Nonetheless, these published reports along with our results from nociceptive assays suggest that the KOP system might be altered in the WKY rat strain and that such alterations may underlie its phenotype. This notion is further supported by findings in our study (Table 7.1) and others (Dennis et al., 2016; Burke et al., 2019) regarding the differential expression of the genes encoding KOP and PDYN in key brain regions implicated in pain and affect in the WKY rats, compared to SD counterparts. Therefore, further investigations examining functional activity of the KOP in these regions are needed. For instance, [^{35}S]GTPγS assay can be employed to assess receptor functionality *in vitro*. In addition, *in vivo* pharmacological studies examining effects of direct intracerebral injections of KOP-modulating drugs into discrete brain regions in the WKY rats could help to elucidate region-dependent role played by the KOP in relation to hyperalgesia and negative affect. A region of interest in this respect could be the amygdala or more specifically the CeA. A recent study has revealed a role for the KOP in the CeA in SIH in a rat model of migraine (Xie et al., 2017). As mentioned earlier, the CeA has dense reciprocal projections with the PAG. Thus, any alteration in the functionality of the KOP in this CeA-PAG pathway may, in turn, affect nociceptive responding in the WKY rats. Besides pharmacological investigation, future studies using electrophysiology could explore KOP signalling/activity in the CeA in WKY and SD rats.

Table 7.1: Summary of results on the expression of genes encoding MOP, KOP, POMC, and PDYN (measured using RT-qPCR) in discrete brain regions involved in pain and affect in naïve WKY rats, compared to SD rats. ↑: higher, ↓: lower, ↔: no difference; Amyg: amygdala, Hypo: hypothalamus, Hipp: hippocampus, KOP: kappa-opioid receptor, MOP: mu-opioid receptor, PAG: periaqueductal grey, PFC: prefrontal cortex, PDYN: prodynorphin, POMC: proopiomelanocortin, SD: Sprague-Dawley, WKY: Wistar-Kyoto.

Receptor/ propeptide	**Regions**				
	PFC	**Amyg**	**Hipp**	**Hypo**	**PAG**
MOP	↔	↔	↑	↔	↔
KOP	↓	↔	↔	↔	↓ (trend)
POMC	↑	↔	↔	↔	↔
PDYN	↑	↔	↑	↔	↓ (trend)

As mentioned earlier in Chapter 1, chronic pain and negative affect share a complex reciprocal relationship. Just as the presence of negative affect can exacerbate pain experience, chronic pain states can also lead to development of anxiety and depressive disorders (McWilliams et al., 2003; Knaster et al., 2012; de Heer et al., 2014), resulting in debilitating comorbidity. In addition to affective disorders, chronic pain is highly comorbid with cognitive impairment in the clinical population (Moriarty et al., 2011; Shin et al., 2012; Moriarty and Finn, 2014; Whitlock et al., 2017). In this respect, I explored different behavioural domains relating to nociception, negative affect, and cognition in the CFA model of chronic inflammatory pain in Chapters 5 and 6.

Results in Chapter 5 provide further evidence for genotype-dependent exacerbation of nociceptive responding in the CFA model of chronic inflammatory pain. While both strains developed mechanical allodynia and thermal hyperalgesia following CFA injection into the hindpaw, WKY rats exhibited greater mechanical allodynia in the VF test, compared to SD counterparts. Similar to our results, others have demonstrated greater mechanical allodynia in the WKY rats after injecting CFA into the temporomandibular joint (Wang et al., 2012) or following chronic constriction nerve injury (Zeng et al., 2008), compared with Wistar rats. Previous work in our laboratory has shown that WKY rats, but not SD counterparts, develop thermal hyperalgesia in the

spinal nerve injury model of neuropathic pain (Jennings, 2015). However, Hestehave et al have reported in two separate studies that the WKY rats develop less robust mechanical hypersensitivity after CFA injection (Hestehave et al., 2019b) or their mechanical hypersensitivity did not differ from SD rats following spared nerve injury (Hestehave et al., 2019a). Differences such as models used to manifest chronic neuropathic pain and behavioural tests employed to assess mechanical hypersensitivity after CFA injection may, at least in part, account for the discrepant results here. Overall, results from our study and the current literature suggest an exacerbation of nociceptive responding following induction of chronic pain in the WKY rat strain.

Furthermore, Chapter 5 examined the effects of CFA-induced chronic inflammatory pain on manifestation of anxiety-like behaviour and cognitive impairment in SD and WKY rats. Strain-related differences were apparent between SD and WKY rats in the measures of activity in a novel aversive environment (an indirect measure for anxiety-like behaviour) and in the domains of social behaviour and recognition and spatial memory. We did not observe pain-induced anxiety or deficits in social and spatial memory in either strain, possibly due to differences in time points of testing between our study and those published (Parent et al., 2012; Grégoire et al., 2014; Wu et al., 2017; Zhong et al., 2019). Therefore, further studies are needed to assess manifestation of anxiety- and depressive-like behaviours, as well as deficits in these cognitive domains, at later time points in the CFA model, and to explore any genotype-dependent exacerbation of these behaviours in the WKY rats. In our study, there was a modest CFA-induced impairment in recognition memory (NOR test) in the SD rats. Similar CFA-induced cognitive impairment has been reported earlier in the NOR test (Amaral et al., 2015) and in other paradigms with learning and memory components (Cain et al., 1997; Baiamonte et al., 2013) in rats. Future studies will need to elucidate the molecular correlates associated with cognitive deficits in the CFA model. Since the opioid system also has a role in learning and memory, it would be interesting to examine any CFA-induced changes in the expression and/or functionality of the different opioid receptors and peptides in key neural substrates (e.g. hippocampus, PFC, PAG) and correlate with behavioural outcome.

The findings from Chapter 5 were followed up with another *in vivo* study presented in Chapter 6 where we further examined the effects of CFA-induced chronic inflammatory pain on the manifestation of any depressive-like behaviour and impairment in recognition memory over time in SD rats. However, in this study we did not observe CFA-induced

impairment in recognition memory in the NOR test at either of the two post-CFA injection time points investigated (Day 18 or 27). One of the differences between the two *in vivo* studies in Chapters 5 and 6 is the aspect of repeated drug/vehicle administration in the latter study. Whether stress from repeated injections has any effect on these behavioural outcomes needs further investigation. A previous study by Amaral and colleagues suggest that CFA injection during neonatal period could have long-lasting detrimental effects on recognition memory in adulthood (Amaral et al., 2015). In addition, there is ample clinical and preclinical evidence indicating that chronic pain induces maladaptive neuroplastic changes in brain regions with overlapping functions in pain, affect, and cognition (Apkarian et al., 2004; Baliki et al., 2006; Mutso et al., 2012; Kiritoshi and Neugebauer, 2015; Nishinaka et al., 2016). Thus, future studies could examine if negative affective states or cognitive impairments manifest beyond the observed chronic inflammatory state.

Comorbidity between chronic pain and affective disorders and/or cognitive impairment results in poor therapeutic outcome for patients. Although there is evidence for a reciprocal relationship between these conditions, the underlying neurobiological mechanisms remain poorly understood, limiting therapeutic options to effectively treat such comorbidity. In this regard, drugs acting at KOP and DOP are currently under active research due to their improved side-effect profile and abuse potential compared to MOP agonists (Al-Hasani and Bruchas, 2011; Wang et al., 2019). KOP antagonists are currently being considered for the treatment of neuropsychiatric diseases, including anxiety, depression, and substance abuse disorders (Carlezon and Krystal, 2016). Recently, KOP antagonists have also gained attention for the treatment of chronic pain (Xie et al., 2017; Liu et al., 2019b; Navratilova et al., 2019). Similarly, DOP agonists display high efficacy in chronic inflammatory pain and migraine headache and have anxiolytic and antidepressant-like effects (Pradhan et al., 2011; Moye et al., 2019). Hence, in Chapter 6, the effects of chronic systemic administration of a KOP antagonist (DIPPA) and a DOP agonist (SNC80) on CFA-induced enhanced nociceptive responding and any associated depressive-like and cognitive deficits were investigated in the SD rats. However, chronic administration of these drugs, alone or in combination, had no overall effect on nociceptive or other behavioural domains in rats, except for a transient DIPPA-induced impairment of recognition memory in the CFA-injected rats. Although the drugs were administered chronically for 30 days, the doses employed in our study may have been too low to elicit a significant pharmacological effect in the behavioural tests. Given

the unique pharmacological profiles of KOP antagonism and DOP agonism, future studies should further explore their therapeutic potential in comorbid chronic pain-negative affect/cognitive impairment and the influence of genetic background thereon.

The studies discussed so far in this thesis have some limitations that should be noted. In particular:

(a) Although every effort has been made to collect data with precision and in a reliable way, we acknowledge the possibility of Type II errors in interpreting experimental datasets. In statistical analysis, Type I (false positive) and Type II (false negative) errors are related in that reducing Type I error tends to increase Type II error and vice-versa (Kim, 2015). We have applied Bonferroni-Holm correction to account for multiple comparisons and, therefore, reduce the probability of making a Type I error. However, in doing so, we could increase chances of committing a Type II error. As such, a power analysis may help to deduce the probability of avoiding a Type II error. This is particularly important in preclinical studies like ours where the sample size is relatively low and it is not always feasible to increase the number of experimental animals as there are ethical implications of using animals for research (Festing and Altman, 2002).

(b) A battery of behavioural tests was employed in the studies following a pharmacological intervention to assess the effects of the drug on multiple behavioural domains. A limitation of such a test battery approach is the impact that stress or handling associated with each task has on the animal. In addition, carryover effects from prior tests may influence behavioural responses in subsequent tests. A pervious study has shown that mice with test battery experience exhibit different behaviour compared to naïve mice (exposed to only one test in the battery) (McIlwain et al., 2001). The authors observed difference in behaviour between test battery and naïve animals for number of tasks including the open field, rotarod, and hot plate tests. Though the use of a test battery approach is a good strategy to obtain most information with the fewest number of animals, the test experience here could be a source of variance in the data.

(c) The studies were conducted using male rats only. Numerous studies have demonstrated sex differences in pain at the clinical level, particularly in chronic pain conditions (Sorge and Totsch, 2017). Despite a higher prevalence of pain reports and disorders in women (Fillingim et al., 2009), preclinical studies in pain have largely used male rodents (Mogil, 2012) that may skew the complete mechanistic picture of chronic pain and any associated affective/cognitive disorders. One of the reasons behind this sex

bias is the commonly held view that different stages of oetrous cycle in female rodents could cause significant variability in behavioural results. However, studies have shown robust sex differences in nociception that are not influenced by fluctuation of hormonal levels at different stages of the oestrous cycle in rats and mice (Mogil et al., 2000). In addition, sex-dependent differences in response to opioids have been reported in both clinical and preclinical studies (Rasakham and Liu-Chen, 2011; Averitt et al., 2019). Though there are some discrepancies, the majority of these studies have demonstrated reduced efficacy of opioids (such as morphine, buprenorphine, nalbuphine, U50488, SNC80) in females compared to males (Sarton et al., 2000; Barrett et al., 2002; Cepeda and Carr, 2003; Aubrun et al., 2005; Stoffel et al., 2005; Bai et al., 2015). Moreover, sex differences in preclinical pain studies have been shown to be dependent on the rodent strain used, and there are strong lines of evidence supporting for a role of genotype in determining sex differences in opioid-mediated antinociception (Mogil et al., 2000; Terner et al., 2003). With regards to the WKY rats, Burke and colleagues has recently demonstrated some sex dimorphism in anxiety- and depression-related behaviours in this strain. Future studies should therefore be carried out using female rats to explore any influence of sex on the genotype-dependent response to opioids in pain-negative affect interaction.

In conclusion, the body of work presented in this thesis further adds to our understanding of altered nociceptive responding in the presence of negative affective state. Our findings extend the behavioural and neurobiological profile of the WKY rat model. The results suggest, for the first time, that deficits in MOP-induced signalling in the vlPAG and consequently impaired engagement of downstream regions of the descending inhibitory pain pathway, may underlie hyperalgesia to noxious persistent inflammatory stimulus in the WKY rat. In addition, WKY rats exhibit differential nociceptive responses to pharmacological modulation of the KOP compared to SD counterparts, suggesting alterations in the KOP system may also account for some of their characteristic behavioural profile. Furthermore, our findings provide evidence for an influence of genotype on exacerbated nociceptive response in chronic inflammatory pain. Lastly, results of the experiments presented herein contribute to our understanding of the complex modulatory effect of chronic pain on cognitive responses.

Appendices

Appendix A: Buffers and solutions – Western immunoblotting

1 M Tris-HCl, pH 7.4

- For 100 mL:
    - Weigh 15.15g Trizma-base (Sigma-Aldrich, Wicklow, Ireland)
    - Add to 50 mL dH_2O and place on stirrer to dissolve
    - Adjust pH to 7.4
    - Make up to a final volume of 100 mL with dH_2O
    - Store at 4ºC

0.5 M Tris-HCl, pH 6.8

- For 200 mL:
    - Weigh 12.11 g Trizma-base
    - Add 50 mL dH_2O and place on stirrer to dissolve
    - Adjust pH to 6.8
    - Make up to a final volume of 200 mL with dH_2O
    - Store at 4ºC

1.5 M Tris-HCl, pH 8.8

- For 500 mL:
    - Weigh 90.82g Trizma-base
    - Add 50 mL dH_2O and place on stirrer to dissolve
    - Adjust pH to 8.8
    - Make up to a final volume of 500 mL with dH_2O
    - Store at 4ºC

RIPA lysis buffer

Ingredients (for 12 mL):	
50 mM EGTA	240 µL
50 mM NaF	120 µL
5 M NaCl	360 µL
1 M Tris-HCl pH 7.4	600 µL
0.25% Na-deoxycholate	300 µL of 10% Na-deoxycholate solution
1 mM Na-orthovanadate	120 µL
dH_2O	12 mL (final volume)

- Male 990 µL aliquots
- Store at -20ºC
- Add 10 µL protease inhibitor cocktail (cat# P8340, Sigma-Aldrich, Wicklow, Ireland) to each aliquot before use

4X Sample buffer

Ingredients (for 50 mL):	
SDS	0.92 g
1 M Tris-HCl pH 6.8	2.5 mL
Glycerol	4 mL
1% Bromophenol Blue (in PBS)	1 mL
dH_2O	50 mL (final volume)

- Make 950 µL aliquots
- Store at -20ºC
- Add 50 µL 2-mercaptoethanol to each aliquot in fume hood when ready to use

10% SDS

- 100 uL 10% SDS (Sigma-Aldrich, Wicklow, Ireland) in 10 mL dH_2O
- Store at room temperature

10% Ammonium persulfate (100 mg/ml)

- Make fresh daily
- As required while making up gels, e.g. 0.05 g in 500 µL

- Store at 4ºC

5X Running buffer

Ingredients (for 1 L):	
Trizma-base	15 g
Glycine	108 g
20% SDS	25 mL
dH_2O	1 L (final volume)

- Store at room temperature
- Dilute to 1X before use

1X Transfer buffer

Ingredients (for 2 L):	
Trizma-base	6.06 g
Glycine	28.8 g
20% Methanol	400 mL
dH_2O	2 L (final volume)

- Store at 4ºC

10X Tris-Buffered Saline (TBS)

Ingredients (for 1 L):	
Trizma-base	30 g
NaCl	80 g
KCl	2 g
dH_2O	1 L (final volume)

- pH to 7.4 before bringing to final volume
- Store at 4ºC
- Dilute to 1X before use

Blocking solution

Ingredients (for 100 mL):	
Non-fat powder milk	5 g
Tween 20	50 µL
1X Tris-buffered saline (TBS)	100 mL (final volume)

- Store at 4ºC

Washing solution

Ingredients (for 100 mL):	
Tween 20	200 µL
1X Tris-buffered saline (TBS)	100 mL (final volume)

- Store at 4ºC

Primary Antibody diluent: stored at 4°C

- Same as blocking solution
- Store at 4ºC

Secondary Antibody diluent

- 1% milk solution (1 g milk in 100 ml TBS with 10 µL of Tween 20)
- Store at 4ºC

Stripping Buffer (25mM Glycine with 1% SDS)

- Add 1.876 g glycine to 950 mL dH_2O
- pH to 2 with HCl
- Add 50 mL of 20% SDS
- Store at room temperature

0.1% Ponceau stain solution

Ingredients (for 100 mL):	
Ponceau stain	100 mg
5% acetic acid	1 mL
dH_2O	100 mL (final volume)

- Store at 4ºC

12% SDS-PAGE Resolving gel (for 2 gels)

Ingredients	Volume
dH_2O	6.58 mL
30% acrylamide mix	8 mL
1.5 M Tris-HCl pH 8.8	5 mL
10% SDS	200 µL
10% ammonium persulphate	200 µL
TEMED	20 µL

4% Stacking gel (for 2 gels)

Ingredients	Volume
dH_2O	5.48 mL
30% acrylamide mix	1.36 mL
0.5 M Tris-HCl pH 6.8	1 mL
10% SDS	80 µL
10% ammonium persulphate	80 µL
TEMED	8 µL

Appendix B: Buffers and solutions – Transcardial perfusion

0.89% w/v NaCl

- Weigh 8.9 g NaCl
- Add deionised water (dH_2O) to make up to 1 L

Heparinised saline

- Add 5,000 units of heparin (Wockhardt Ltd, Wrexham, UK) per litre of 0.89% NaCl

Cryoprotective solution (25% w/v sucrose solution (1 L) with 0.1% sodium azide)

- 0.1 M PBS: Dissolve 5 PBS tablets (Sigma-Aldrich, Wicklow, Ireland) in 500 mL
- Add 250 g of sucrose (Fisher Scientific, Leicestershire, UK)
- Add 1 g sodium azide (Sigma-Aldrich, Wicklow, Ireland)
- Stir until dissolved
- Make up to 1 L with dH_2O

Appendix C: Buffers and solutions – Immunohistochemistry

0.1% w/v PB-Azide

- 0.1 M PB:
    - Weigh 3.1188 g of $NaH_2PO_4.H_2O$ (stock A) (Sigma-Aldrich, Wicklow, Ireland)
    - Weigh 13.7772 g of $Na_2HPO_4.2H_2O$ (stock B) (Fisher Scientific, Leicestershire, UK)
    - Add dH_2O to make up to 1 L
- Weigh out 1 g sodium azide
- Add to 1 L of 0.1 M PB and stir to dissolve

Quench

- 30% H_2O_2 (Sigma-Aldrich, Wicklow, Ireland) in 10% methanol/dH_2O (Fisher Scientific, Leicestershire, UK)

Blocking solution

- 0.2% TXPBS: Add 3 mL of Triton-X (Sigma-Aldrich, Wicklow, Ireland) to 1 L of 0.1 M PBS
- 3% normal goat serum (NGS) (Abcam, Cambridge, UK) in 0.2% TXPBS

Primary Antibody

- Rabbit anti-cFos (1:2000, cat#ab190289, Abcam, Cambridge, UK) in 1% NGS in 0.2% TXPBS

Secondary Antibody

- Biotinylated goat anti-rabbit antisera (1:200, cat#ab97049, Abcam, Cambridge, UK) in 1% NGS in 0.1 M PBS

References

Abbadie, C., Taylor, B.K., Peterson, M.A., and Basbaum, A.I. (1997). Differential contribution of the two phases of the formalin test to the pattern of c-fos expression in the rat spinal cord: studies with remifentanil and lidocaine. Pain *69*: 101–110.

Abood, M.E., Noel, M.A., Farnsworth, J.S., and Tao, Q. (1994). Molecular cloning and expression of a delta-opioid receptor from rat brain. J. Neurosci. Res. *37*: 714–719.

Ackerman, A.L., Jellison, F.C., Lee, U.J., Bradesi, S., and Rodriguez, L. V (2016). The Glt1 glutamate receptor mediates the establishment and perpetuation of chronic visceral pain in an animal model of stress-induced bladder hyperalgesia. Am. J. Physiol. Renal Physiol. *310*: F628–F636.

Agostini, S., Eutamene, H., Broccardo, M., Improta, G., Petrella, C., Theodorou, V., et al. (2009). Peripheral anti-nociceptive effect of nociceptin/orphanin FQ in inflammation and stress-induced colonic hyperalgesia in rats. Pain *141*: 292–299.

Aguggia, M. (2003). Neurophysiology of pain. Neurol. Sci. *24 Suppl 2*: S57-60.

Ait-Belgnaoui, A., Eutamene, H., Houdeau, E., Bueno, L., Fioramonti, J., and Theodorou, V. (2009). Lactobacillus farciminis treatment attenuates stress-induced overexpression of Fos protein in spinal and supraspinal sites after colorectal distension in rats. Neurogastroenterol. Motil. *21*: 567–73, e18-9.

Akagi, T., Matsumura, Y., Yasui, M., Minami, E., Inoue, H., Masuda, T., et al. (2014). Interferon regulatory factor 8 expressed in microglia contributes to tactile allodynia induced by repeated cold stress in rodents. J. Pharmacol. Sci. *126*: 172–176.

Akil, H., Mayer, D.J., and Liebeskind, J.C. (1972). Comparison in the rat between analgesia induced by stimulation of periacqueducal gray matter and morphine analgesia. C. R. Acad. Sci. Hebd. Seances Acad. Sci. D. *274*: 3603–3605.

Akil, H., Mayer, D.J., and Liebeskind, J.C. (1976). Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. Science *191*: 961–2.

Akinfiresoye, L., and Tizabi, Y. (2013). Antidepressant effects of AMPA and ketamine combination: role of hippocampal BDNF, synapsin, and mTOR. Psychopharmacology (Berl). *230*: 291–298.

Al-Hasani, R., and Bruchas, M.R. (2011). Molecular mechanisms of opioid receptor-dependent signaling and behavior. Anesthesiology *115*: 1363–1381.

Alba-Delgado, C., Llorca-Torralba, M., Horrillo, I., Ortega, J.E., Mico, J.A., Sánchez-Blázquez, P., et al. (2013). Chronic pain leads to concomitant noradrenergic impairment and mood disorders. Biol. Psychiatry *73*: 54–62.

Albe-Fessard, D., Berkley, K.J., Kruger, L., Ralston, H.J. 3rd, and Willis, W.D.J. (1985). Diencephalic mechanisms of pain sensation. Brain Res. *356*: 217–296.

Albert-Vartanian, A., Boyd, M.R., Hall, A.L., Morgado, S.J., Nguyen, E., Nguyen, V.P.H., et al. (2016). Will peripherally restricted kappa-opioid receptor agonists (pKORAs) relieve pain with less opioid adverse effects and abuse potential? J. Clin. Pharm. Ther. *41*: 371–382.

Almatroudi, A., Husbands, S.M., Bailey, C.P., and Bailey, S.J. (2015). Combined administration of buprenorphine and naltrexone produces antidepressant-like effects in mice. J. Psychopharmacol. *29*: 812–821.

Almay, B.G., Johansson, F., Knorring, L. Von, Terenius, L., and Wahlstrom, A. (1978). Endorphins in chronic pain. I. Differences in CSF endorphin levels between organic and psychogenic pain syndromes. Pain *5*: 153–162.

Almeida, T.F., Roizenblatt, S., and Tufik, S. (2004). Afferent pain pathways: A neuroanatomical review. Brain Res. *1000*: 40–56.

Amaral, C., Antonio, B., Oliveira, M.G.M., Hamani, C., Guinsburg, R., and Covolan, L. (2015). Early postnatal nociceptive stimulation results in deficits of spatial memory in male rats. Neurobiol. Learn. Mem. *125*: 120–125.

Amiri, S., Amini-Khoei, H., Mohammadi-Asl, A., Alijanpour, S., Haj-Mirzaian, A., Rahimi-Balaei, M., et al. (2016). Involvement of D1 and D2 dopamine receptors in the antidepressant-like effects of selegiline in maternal separation model of mouse. Physiol. Behav. *163*: 107–114.

Amorim, D., David-Pereira, A., Pertovaara, A., Almeida, A., and Pinto-Ribeiro, F. (2014). Amitriptyline reverses hyperalgesia and improves associated mood-like disorders in a model of experimental monoarthritis. Behav. Brain Res. *265*: 12–21.

An, X., Bandler, R., Ongur, D., and Price, J.L. (1998). Prefrontal cortical projections to longitudinal columns in the midbrain periaqueductal gray in macaque monkeys. J. Comp. Neurol. *401*: 455–479.

Andero, R. (2015). Nociceptin and the nociceptin receptor in learning and memory. Prog. Neuro-Psychopharmacology Biol. Psychiatry *62*: 45–50.

Anderson, R.I., Morales, M., Spear, L.P., and Varlinskaya, E.I. (2014). Pharmacological activation of kappa opioid receptors: aversive effects in adolescent and adult male rats. Psychopharmacology (Berl). *231*: 1687–1693.

Anseloni, V.C., Coimbra, N.C., Morato, S., and Brandao, M.L. (1999). A comparative study of the effects of morphine in the dorsal periaqueductal gray and nucleus accumbens of rats submitted to the elevated plus-maze test. Exp. Brain Res. *129*: 260–268.

Apkarian, A.V., Sosa, Y., Sonty, S., Levy, R.M., Harden, R.N., Parrish, T.B., et al. (2004). Chronic back pain is associated with decreased prefrontal and thalamic gray matter density. J. Neurosci. *24*: 10410–10415.

Apkarian, A.V., Thomas, P.S., Krauss, B.R., and Szeverenyi, N.M. (2001). Prefrontal cortical hyperactivity in patients with sympathetically mediated chronic pain. Neurosci. Lett. *311*: 193–197.

Asmundson, G.J.G., and Katz, J. (2009). Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. Depress. Anxiety *26*: 888–901.

Atkinson, J.H., Kremer, E.F., Risch, S.C., Morgan, C.D., Azad, R.F., Ehlers, C.L., et al. (1983). Plasma measures of beta-endorphin/beta-lipotropin-like immunoreactivity in chronic pain syndrome and psychiatric subjects. Psychiatry Res. *9*: 319–327.

Attali, B., and Vogel, Z. (1986). Inhibition of adenylate cyclase and induction of heterologous desensitization by kappa agonists in rat spinal cord. NIDA Res. Monogr. *75*: 141–144.

Aubrun, F., Salvi, N., Coriat, P., and Riou, B. (2005). Sex- and age-related differences in morphine requirements for postoperative pain relief. Anesthesiology *103*: 156–160.

Augustine, J.R. (1996). Circuitry and functional aspects of the insular lobe in primates including humans. Brain Res. Rev. *22*: 229–244.

Averitt, D.L., Eidson, L.N., Doyle, H.H., and Murphy, A.Z. (2019). Neuronal and glial factors contributing to sex differences in opioid modulation of pain. Neuropsychopharmacology *44*: 155–165.

Avila-Martin, G., Galan-Arriero, I., Ferrer-Donato, A., Busquets, X., Gomez-Soriano, J., Escriba, P. V, et al. (2015). Oral 2-hydroxyoleic acid inhibits reflex hypersensitivity and open-field-induced anxiety after spared nerve injury. Eur. J. Pain *19*: 111–122.

Avsaroglu, H., Sar, A.S. van der, Lith, H.A. van, Zutphen, L.F.M. van, and Hellebrekers, L.J. (2007). Differences in response to anaesthetics and analgesics between inbred rat strains. Lab. Anim. *41*: 337–344.

Bagdas, D., Muldoon, P.P., Alsharari, S., Carroll, F.I., Negus, S.S., and Damaj, M.I. (2016). Expression and pharmacological modulation of visceral pain-induced conditioned place aversion in mice. Neuropharmacology *102*: 236–243.

Bagnol, D., Mansour, A., Akil, H., and Watson, S.J. (1997). Cellular localization and distribution of the cloned mu and kappa opioid receptors in rat gastrointestinal tract. Neuroscience *81*: 579–591.

Bai, X., Zhang, X., Li, Y., Lu, L., Li, B., and He, X. (2015). Sex differences in peripheral mu-opioid receptor mediated analgesia in rat orofacial persistent pain model. PLoS One *10*: e0122924.

Baiamonte, B.A., Lee, F.A., Gould, H.J. 3rd, and Soignier, R.D. (2013). Morphine-induced cognitive impairment is attenuated by induced pain in rats. Behav. Neurosci. *127*: 524–534.

Bair, M.J., Poleshuck, E.L., Wu, J., Krebs, E.K., Damush, T.M., Tu, W., et al. (2013). Anxiety but not social stressors predict 12-month depression and pain severity. Clin. J. Pain *29*: 95–101.

Bair, M.J., Robinson, R.L., Katon, W., and Kroenke, K. (2003). Depression and pain comorbidity: a literature review. Arch. Intern. Med. *163*: 2433–2445.

Bair, M.J., Wu, J., Damush, T.M., Sutherland, J.M., and Kroenke, K. (2008). Association of depression and anxiety alone and in combination with chronic musculoskeletal pain in primary care patients. Psychosom. Med. *70*: 890–897.

Baliki, M.N., Chialvo, D.R., Geha, P.Y., Levy, R.M., Harden, R.N., Parrish, T.B., et al. (2006). Chronic pain and the emotional brain: specific brain activity associated with spontaneous fluctuations of intensity of chronic back pain. J. Neurosci. *26*: 12165–12173.

Bals-Kubik, R., Ableitner, A., Herz, A., and Shippenberg, T.S. (1993). Neuroanatomical sites mediating the motivational effects of opioids as mapped by the conditioned place preference paradigm in rats. J. Pharmacol. Exp. Ther. *264*: 489–495.

Bandler, R., and Keay, K.A. (1996). Chapter 17 Columnar organization in the midbrain periaqueductal gray and the integration of emotional expression. In Progress in Brain Research *107*: 285–300.

Bandler, R., and Shipley, M.T. (1994). Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? Trends Neurosci. *17*: 379–389.

Bannon, A.W., and Malmberg, A.B. (2007). Models of nociception: hot-plate, tail-flick, and formalin tests in rodents. Curr. Protoc. Neurosci. *Chapter 8*: Unit 8.9.

Bar, K.J., Brehm, S., Boettger, M.K., Boettger, S., Wagner, G., and Sauer, H. (2005). Pain perception in major depression depends on pain modality. Pain *117*: 97–103.

Barbaro, N.M., Heinricher, M.M., and Fields, H.L. (1986). Putative pain modulating neurons in the rostral ventral medulla: Reflex-related activity predicts effects of morphine. Brain Res. *366*: 203–210.

Barkus, C., McHugh, S.B., Sprengel, R., Seeburg, P.H., Rawlins, J.N.P., and Bannerman, D.M. (2010). Hippocampal NMDA receptors and anxiety: At the interface between cognition and emotion. Eur. J. Pharmacol. *626*: 49–56.

Baron, R., Hans, G., and Dickenson, A.H. (2013). Peripheral input and its importance for central sensitization. Ann. Neurol. *74*: 630–636.

Baronio, D., Castro, K., Gonchoroski, T., Melo, G.M. de, Nunes, G.D.F., Bambini-Junior, V., et al. (2015). Effects of an H3R antagonist on the animal model of autism induced by prenatal exposure to valproic acid. PLoS One *10*: e0116363.

Barrett, A.C., Smith, E.S., and Picker, M.J. (2002). Sex-related differences in mechanical nociception and antinociception produced by μ- and κ-opioid receptor agonists in rats. Eur. J. Pharmacol. *452*: 163–173.

Barrot, M. (2012). Tests and models of nociception and pain in rodents. Neuroscience *211*: 39–50.

Basbaum, A.I., Bautista, D.M., Scherrer, G., and Julius, D. (2009). Cellular and molecular mechanisms of pain. Cell *139*: 267–84.

Basbaum, A.I., Clanton, C.H., and Fields, H.L. (1976). Opiate and stimulus-produced analgesia: functional anatomy of a medullospinal pathway. Proc. Natl. Acad. Sci. U. S. A. *73*: 4685–4688.

Basbaum, A.I., Clanton, C.H., and Fields, H.L. (1978). Three bulbospinal pathways from the rostral medulla of the cat: an autoradiographic study of pain modulating systems. J. Comp. Neurol. *178*: 209–224.

Basbaum, A.I., and Fields, H.L. (1979). The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. J. Comp. Neurol. *187*: 513–531.

Basbaum, A.I., and Fields, H.L. (1984). Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu. Rev. Neurosci. *7*: 309–38.

Baxter, M.G., and Murray, E.A. (2002). The amygdala and reward. Nat. Rev. Neurosci. *3*: 563–573.

Beardsley, P.M., Howard, J.L., Shelton, K.L., and Carroll, F.I. (2005). Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats. Psychopharmacology (Berl). *183*: 118–126.

Bederson, J.B., Fields, H.L., and Barbaro, N.M. (1990). Hyperalgesia during naloxone-precipitated withdrawal from morphine is associated with increased on-cell activity in the rostral ventromedial medulla. Somatosens. Mot. Res. *7*: 185–203.

Beesdo, K., Jacobi, F., Hoyer, J., Low, N.C.P., Hofler, M., and Wittchen, H.-U. (2010). Pain associated with specific anxiety and depressive disorders in a nationally representative population sample. Soc. Psychiatry Psychiatr. Epidemiol. *45*: 89–104.

Befort, K., Mattei, M.G., Roeckel, N., and Kieffer, B. (1994). Chromosomal localization of the δ opioid receptor gene to human 1p34.3- p36.1 and mouse 4d bands by in situ hybridization. Genomics *20*: 143–145.

Beggs, S., and Salter, M.W. (2013). The known knowns of microglia-neuronal signalling in neuropathic pain. Neurosci. Lett. *557*: 37–42.

Behbehani, M.M. (1995). Functional characteristics of the midbrain periaqueductal gray. Prog. Neurobiol. *46*: 575–605.

Beitz, A.J. (1985). The midbrain periaqueductal gray in the rat. I. Nuclear volume, cell number, density, orientation, and regional subdivisions. J. Comp. Neurol. *237*: 445–459.

Beitz, A.J., Mullett, M.A., and Weiner, L.L. (1983). The periaqueductal gray projections to the rat spinal trigeminal, raphe magnus, gigantocellular pars alpha and paragigantocellular nuclei arise from separate neurons. Brain Res. *288*: 307–314.

Belcheva, I., Ivanova, M., Tashev, R., and Belcheva, S. (2009). Differential involvement of hippocampal vasoactive intestinal peptide in nociception of rats with a model of depression. Peptides *30*: 1497–1501.

Benarroch, E.E. (2012a). Endogenous opioid systems: current concepts and clinical correlations. Neurology *79*: 807–814.

Benarroch, E.E. (2012b). Periaqueductal gray: an interface for behavioural control. Neurology *78*: 210–217.

Benbouzid, M., Pallage, V., Rajalu, M., Waltisperger, E., Doridot, S., Poisbeau, P., et al. (2008). Sciatic nerve cuffing in mice: A model of sustained neuropathic pain. Eur. J. Pain *12*: 591–599.

Berger, B., Rothmaier, A.K., Wedekind, F., Zentner, J., Feuerstein, T.J., and Jackisch, R. (2006). Presynaptic opioid receptors on noradrenergic and serotonergic neurons in the human as compared to the rat neocortex. Br. J. Pharmacol. *148*: 795–806.

Bernard, J.F., Bester, H., and Besson, J.M. (1996). Involvement of the spino-parabrachio -amygdaloid and -hypothalamic pathways in the autonomic and affective emotional aspects of pain. Prog. Brain Res. *107*: 243–255.

Bernardi, M., Genedani, S., Tagliavini, S., and Bertolini, A. (1986). Effects on long-term sensitivity to pain and morphine of stress induced in the newborn rat by pain or manipulation. Physiol. Behav. *37*: 827–831.

Berrocoso, E., Ikeda, K., Sora, I., Uhl, G.R., Sanchez-Blazquez, P., and Mico, J.A. (2013). Active behaviours produced by antidepressants and opioids in the mouse tail suspension test. Int. J. Neuropsychopharmacol. *16*: 151–162.

Berthele, A., Platzer, S., Dworzak, D., Schadrack, J., Mahal, B., Büttner, A., et al. (2003). [3H]-nociceptin ligand-binding and nociceptin opioid receptor mRNA expression in the human brain. Neuroscience *121*: 629–640.

Berton, O., Ramos, A., Chaouloff, F., and Mormde, P. (1997). Behavioral reactivity to social and nonsocial stimulations: a multivariate analysis of six inbred rat strains. Behav. Genet. *27*: 155–166.

Besson, A., Privat, A.M., Eschalier, A., and Fialip, J. (1996). Effects of morphine, naloxone and their interaction in the learned-helplessness paradigm in rats. Psychopharmacology (Berl). *123*: 71–78.

Bester, H., Matsumoto, N., Besson, J.M., and Bernard, J.F. (1997). Further evidence for the involvement of the spinoparabrachial pathway in nociceptive processes: a c-Fos study in the rat. J. Comp. Neurol. *383*: 439–458.

Beutler, L.E., Daldrup, R.J., Engle, D., Elena Oró-Beutler, M., Meredith, K., and Boyer, J.T. (1987). Effects of therapeutically induced affect arousal on depressive symptoms, pain and beta-endorphins among rheumatoid arthritis patients. Pain *29*: 325–334.

Bhargava, H.N. (1982). Pharmacological responses to acute morphine administration in normotensive Wistar-Kyoto and spontaneously hypertensive rats. Life Sci. *31*: 2463–2470.

Bhargava, H.N., and Villar, V.M. (1992). Pharmacodynamics and pharmacokinetics of intravenously administered morphine in spontaneously hypertensive and normotensive Wistar-Kyoto rats. J. Pharmacol. Exp. Ther. *261*: 290–296.

Bianchi, M., and Panerai, A.E. (1993). Naloxone-induced analgesia: Involvement of κ-opiate receptors. Pharmacol. Biochem. Behav. *46*: 145–148.

Biglarnia, M., Karami, M., and Hafshejani, Z.K. (2013). Differences in morphine-induced antinociception in male and female offspring born of morphine exposed mothers. Indian J. Pharmacol. *45*: 227–231.

Bilkei-Gorzo, A., Mauer, D., Michel, K., and Zimmer, A. (2014). Dynorphins regulate the strength of social memory. Neuropharmacology *77*: 406–413.

Blanchard, E.B., Lackner, J.M., Jaccard, J., Rowell, D., Carosella, A.M., Powell, C., et al. (2008). The role of stress in symptom exacerbation among IBS patients. J. Psychosom. Res. *64*: 119–128.

Blankstein, U., Chen, J., Diamant, N.E., and Davis, K.D. (2010). Altered Brain Structure in Irritable Bowel Syndrome: Potential Contributions of Pre-Existing and Disease-Driven Factors. Gastroenterology *138*: 1783–1789.

Bliss, T. V, and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361*: 31–39.

Blumberg, H., Dayton, H.B., George, M., and Rapaport, D. (1961). N-Allyl-noroxymorphone: A potent narcotic antagonist. Fed. Proc. *20*: 311.

Bobeck, E.N., Haseman, R.A., Hong, D., Ingram, S.L., and Morgan, M.M. (2012). Differential development of antinociceptive tolerance to morphine and fentanyl is not linked to efficacy in the ventrolateral periaqueductal gray of the rat. J. Pain *13*: 799–807.

Bobeck, E.N., McNeal, A.L., and Morgan, M.M. (2009). Drug dependent sex-differences in periaqueducatal gray mediated antinociception in the rat. Pain *147*: 210–216.

Bodnar, R.J., Williams, C.L., Lee, S.J., and Pasternak, G.W. (1988). Role of μ1-opiate receptors in supraspinal opiate analgesia: a microinjection study. Brain Res. *447*: 25–34.

Bolles, R.C., and Fanselow, M.S. (1980). A perceptual-defensive-recuperative model of fear and pain. Behav. Brain Sci. *3*: 291–301.

Borges, G., Neto, F., Mico, J.A., and Berrocoso, E. (2014). Reversal of monoarthritis-induced affective disorders by diclofenac in rats. Anesthesiology *120*: 1476–1490.

Borsook, D., Hargreaves, R., Bountra, C., and Porreca, F. (2014). Lost but making progress-Where will new analgesic drugs come from? Sci. Transl. Med. *6*: 249sr3.

Boules, M., Johnston, H., Tozy, J., Smith, K., Li, Z., and Richelson, E. (2011). Analgesic synergy of neurotensin receptor subtype 2 agonist NT79 and morphine. Behav. Pharmacol. *22*: 573–581.

Bourgeais, L., Gauriau, C., and Bernard, J.F. (2001). Projections from the nociceptive

area of the central nucleus of the amygdala to the forebrain: a PHA-L study in the rat. Eur. J. Neurosci. *14*: 229–255.

Boyce-Rustay, J.M., Zhong, C., Kohnken, R., Baker, S.J., Simler, G.H., Wensink, E.J., et al. (2010). Comparison of mechanical allodynia and the affective component of inflammatory pain in rats. Neuropharmacology *58*: 537–543.

Breasted, J. (1930). Ancient Records of Egypt (Chicago: University of Chicago Press).

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., and Gallacher, D. (2006). Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. Eur. J. Pain *10*: 287–333.

Breivik, H., Eisenberg, E., and O'Brien, T. (2013). The individual and societal burden of chronic pain in Europe: the case for strategic prioritisation and action to improve knowledge and availability of appropriate care. BMC Public Health *13*: 1229.

Broom, D.C., Jutkiewicz, E.M., Folk, J.E., Traynor, J.R., Rice, K.C., and Woods, J.H. (2002). Nonpeptidic delta-opioid receptor agonists reduce immobility in the forced swim assay in rats. Neuropsychopharmacology *26*: 744–755.

Brown, A.G. (1981). The spinocervical tract. Prog. Neurobiol. *17*: 59–96.

Browne, C.A., and Lucki, I. (2019). Targeting opioid dysregulation in depression for the development of novel therapeutics. Pharmacol. Ther. *201*: 51–76.

Browne, C.A., Nest, D.S. van, and Lucki, I. (2015). Antidepressant-like effects of buprenorphine in rats are strain dependent. Behav. Brain Res. *278*: 385–392.

Bruchas, M.R., Land, B.B., Aita, M., Xu, M., Barot, S.K., Li, S., et al. (2007). Stress-induced p38 mitogen-activated protein kinase activation mediates kappa-opioid-dependent dysphoria. J. Neurosci. *27*: 11614–11623.

Bruchas, M.R., Macey, T.A., Lowe, J.D., and Chavkin, C. (2006). Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin-dependent in neurons and astrocytes. J. Biol. Chem. *281*: 18081–18089.

Bucher, B., Bettermann, R., and Illes, P. (1987). Plasma concentration and vascular effect of beta-endorphin in spontaneously hypertensive and Wistar Kyoto rats. Naunyn. Schmiedebergs. Arch. Pharmacol. *335*: 428–432.

Buckley, M.M., O'Halloran, K.D., Rae, M.G., Dinan, T.G., and O'Malley, D. (2014). Modulation of enteric neurons by interleukin-6 and corticotropin-releasing factor contributes to visceral hypersensitivity and altered colonic motility in a rat model of irritable bowel syndrome. J. Physiol. *592*: 5235–5250.

Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M., et al. (1994). Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. FEBS Lett. *347*: 284–288.

Bura, A.S., Guegan, T., Zamanillo, D., Vela, J.M., and Maldonado, R. (2013). Operant self-administration of a sigma ligand improves nociceptive and emotional manifestations of neuropathic pain. Eur. J. Pain *17*: 832–843.

Buritova, J., Besson, J.M., and Bernard, J.F. (1998). Involvement of the spinoparabrachial pathway in inflammatory nociceptive processes: a c-Fos protein study in the awake rat. J. Comp. Neurol. *397*: 10–28.

Burke, N.N., Coppinger, J., Deaver, D.R., Roche, M., Finn, D.P., and Kelly, J. (2016). Sex differences and similarities in depressive- and anxiety-like behaviour in the Wistar-Kyoto rat. Physiol. Behav. *167*: 28–34.

Burke, N.N., Ferdousi, M., Deaver, D.R., Finn, D.P., Roche, M., and Kelly, J.P. (2019). Locomotor and anti-immobility effects of buprenorphine in combination with the opioid receptor modulator samidorphan in rats. Neuropharmacology *146*: 327–336.

Burke, N.N., Geoghegan, E., Kerr, D.M., Moriarty, O., Finn, D.P., and Roche, M. (2013a). Altered neuropathic pain behaviour in a rat model of depression is associated with changes in inflammatory gene expression in the amygdala. Genes. Brain. Behav. *12*: 705–713.

Burke, N.N., Hayes, E., Calpin, P., Kerr, D.M., Moriarty, O., Finn, D.P., et al. (2010). Enhanced nociceptive responding in two rat models of depression is associated with alterations in monoamine levels in discrete brain regions. Neuroscience *171*: 1300–1313.

Burke, N.N., Kerr, D.M., Moriarty, O., Finn, D.P., and Roche, M. (2014). Minocycline modulates neuropathic pain behaviour and cortical M1-M2 microglial gene expression in a rat model of depression. Brain. Behav. Immun. *42*: 147–156.

Burke, N.N., Llorente, R., Marco, E.M., Tong, K., Finn, D.P., Viveros, M.P., et al. (2013b). Maternal deprivation is associated with sex-dependent alterations in nociceptive behavior and neuroinflammatory mediators in the rat following peripheral nerve injury. J. Pain *14*: 1173–1184.

Burma, N.E., Leduc-Pessah, H., Fan, C.Y., and Trang, T. (2017). Animal models of chronic pain: Advances and challenges for clinical translation. J. Neurosci. Res. *95*: 1242–1256.

Burns, J.W., Bruehl, S., France, C.R., Schuster, E., Orlowska, D., Buvanendran, A., et al. (2017). Psychosocial factors predict opioid analgesia through endogenous opioid function. Pain *158*: 391–399.

Burston, J.J., Valdes, A.M., Woodhams, S.G., Mapp, P.I., Stocks, J., Watson, D.J.G., et al. (2019). The impact of anxiety on chronic musculoskeletal pain and the role of astrocyte activation. Pain *160*: 658–669.

Burton, M.B., and Gebhart, G.F. (1998). Effects of kappa-opioid receptor agonists on responses to colorectal distension in rats with and without acute colonic inflammation. J. Pharmacol. Exp. Ther. *285*: 707–715.

Buscemi, V., Chang, W.J., Liston, M.B., McAuley, J.H., and Schabrun, S.M. (2019). The Role of Perceived Stress and Life Stressors in the Development of Chronic Musculoskeletal Pain Disorders: A Systematic Review. J. Pain *20*: 1127–1139.

Bushnell, M.C., Čeko, M., and Low, L.A. (2013). Cognitive and emotional control of pain and its disruption in chronic pain. Nat. Rev. Neurosci. *14*: 502–511.

Butelman, E.R., McElroy, B.D., Prisinzano, T.E., and Kreek, M.J. (2019). Impact of pharmacological manipulation of the κ-opioid receptor system on self-grooming and anhedonic-like behaviors in male mice. J. Pharmacol. Exp. Ther. *370*: 1–8.

Butler, R.K., and Finn, D.P. (2009). Stress-induced analgesia. Prog. Neurobiol. *88*: 184–202.

Cahill, C.M., Morinville, A., Hoffert, C., O'Donnell, D., and Beaudet, A. (2003). Up-regulation and trafficking of delta opioid receptor in a model of chronic inflammation:

implications for pain control. Pain *101*: 199–208.

Cahill, C.M., Taylor, A.M.W., Cook, C., Ong, E., Morón, J.A., and Evans, C.J. (2014). Does the kappa opioid receptor system contribute to pain aversion? Front. Pharmacol. *5*: 253.

Cain, C.K., Francis, J.M., Plone, M.A., Emerich, D.F., and Lindner, M.D. (1997). Pain-related disability and effects of chronic morphine in the adjuvant-induced arthritis model of chronic pain. Physiol. Behav. *62*: 199–205.

Calejesan, A.A., Kim, S.J., and Zhuo, M. (2000). Descending facilitatory modulation of a behavioral nociceptive response by stimulation in the adult rat anterior cingulate cortex. Eur. J. Pain *4*: 83–96.

Callaghan, C.K., Rouine, J., Dean, R.L., Knapp, B.I., Bidlack, J.M., Deaver, D.R., et al. (2018). Antidepressant-like effects of 3-carboxamido seco-nalmefene (3CS-nalmefene), a novel opioid receptor modulator, in a rat IFN-α-induced depression model. Brain. Behav. Immun. *67*: 152–162.

Cameron, A.A., Khan, I.A., Westlund, K.N., and Willis, W.D. (1995). The efferent projections of the periaqueductal gray in the rat: a Phaseolus vulgaris-leucoagglutinin study. II. Descending projections. J. Comp. Neurol. *351*: 585–601.

Campion, K.N., Saville, K.A., and Morgan, M.M. (2016). Relative contribution of the dorsal raphe nucleus and ventrolateral periaqueductal gray to morphine antinociception and tolerance in the rat. Eur. J. Neurosci. *44*: 2667–2672.

Cannon, J.T., Prieto, G.J., Lee, A., and Liebeskind, J.C. (1982). Evidence for opioid and non-opioid forms of stimulation-produced analgesia in the rat. Brain Res. *243*: 315–321.

Cao, C.Q., Hong, Y., Dray, A., and Perkins, M. (2001). Spinal δ-opioid receptors mediate suppression of systemic SNC80 on excitability of the flexor reflex in normal and inflamed rat. Eur. J. Pharmacol. *418*: 79–87.

Caputi, F.F., Rullo, L., Stamatakos, S., Candeletti, S., and Romualdi, P. (2019). Modulation of the Negative Affective Dimension of Pain: Focus on Selected Neuropeptidergic System Contributions. Int. J. Mol. Sci. *20*: 4010.

Carey, A.N., Lyons, A.M., Shay, C.F., Dunton, O., and McLaughlin, J.P. (2009). Endogenous kappa opioid activation mediates stress-induced deficits in learning and memory. J. Neurosci. *29*: 4293–300.

Carlezon, W.A., and Krystal, A.D. (2016). Kappa-Opioid Antagonists for Psychiatric Disorders: From Bench to Clinical Trials. Depress. Anxiety *33*: 895–906.

Carlezon, W.A.J., Beguin, C., DiNieri, J.A., Baumann, M.H., Richards, M.R., Todtenkopf, M.S., et al. (2006). Depressive-like effects of the kappa-opioid receptor agonist salvinorin A on behavior and neurochemistry in rats. J. Pharmacol. Exp. Ther. *316*: 440–447.

Carr, G. V, Bangasser, D.A., Bethea, T., Young, M., Valentino, R.J., and Lucki, I. (2010). Antidepressant-like effects of kappa-opioid receptor antagonists in Wistar Kyoto rats. Neuropsychopharmacology *35*: 752–63.

Carr, G. V, and Lucki, I. (2010). Comparison of the kappa-opioid receptor antagonist DIPPA in tests of anxiety-like behavior between Wistar Kyoto and Sprague Dawley rats. Psychopharmacology (Berl). *210*: 295–302.

Casey, K.L., Minoshima, S., Berger, K.L., Koeppe, R.A., Morrow, T.J., and Frey, K.A.

(1994). Positron emission tomographic analysis of cerebral structures activated specifically by repetitive noxious heat stimuli. J. Neurophysiol. *71*: 802–807.

Caspani, O., Reitz, M.C., Ceci, A., Kremer, A., and Treede, R.D. (2014). Tramadol reduces anxiety-related and depression-associated behaviors presumably induced by pain in the chronic constriction injury model of neuropathic pain in rats. Pharmacol. Biochem. Behav. *124*: 290–296.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., et al. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science *288*: 306–313.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature *389*: 816–824.

Cepeda, M.S., and Carr, D.B. (2003). Women experience more pain and require more morphine than men to achieve a similar degree of analgesia. Anesth. Analg. *97*: 1464–1468.

Chan, S.W.Y., Harmer, C.J., Norbury, R., O'Sullivan, U., Goodwin, G.M., and Portella, M.J. (2016). Hippocampal volume in vulnerability and resilience to depression. J. Affect. Disord. *189*: 199–202.

Chang, A.C., Takemori, A.E., Ojala, W.H., Gleason, W.B., and Portoghese, P.S. (1994a). κ Opioid Receptor Selective Affinity Labels: Electrophilic Benzeneacetamides as κ Selective Opioid Antagonists. J. Med. Chem. *37*: 4490–4498.

Chang, A.C., Takemori, A.E., and Portoghese, P.S. (1994b). 2-(3,4-Dichlorophenyl)-N-methyl-N-[(1S)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidi nyl)ethyl]acetamide: an opioid receptor affinity label that produces selective and long-lasting kappa antagonism in mice. J. Med. Chem. *37*: 1547–1549.

Chao, C.C., Gekker, G., Hu, S., Sheng, W.S., Shark, K.B., Bu, D.F., et al. (1996). kappa opioid receptors in human microglia downregulate human immunodeficiency virus 1 expression. Proc. Natl. Acad. Sci. U. S. A. *93*: 8051–8056.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., and Yaksh, T.L. (1994). Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods *53*: 55–63.

Chavkin, C., James, I.F., and Goldstein, A. (1982). Dynorphin is a specific endogenous ligand of the kappa opioid receptor. Science *215*: 413–415.

Chefer, V.I., Backman, C.M., Gigante, E.D., and Shippenberg, T.S. (2013). Kappa opioid receptors on dopaminergic neurons are necessary for kappa-mediated place aversion. Neuropsychopharmacology *38*: 2623–2631.

Chen, F.L., Dong, Y.L., Zhang, Z.J., Cao, D.L., Xu, J., Hui, J., et al. (2012). Activation of astrocytes in the anterior cingulate cortex contributes to the affective component of pain in an inflammatory pain model. Brain Res. Bull. *87*: 60–66.

Chen, J., and Hackos, D.H. (2015). TRPA1 as a drug target--promise and challenges. Naunyn. Schmiedebergs. Arch. Pharmacol. *388*: 451–463.

Chen, J., Song, Y., Yang, J., Zhang, Y., Zhao, P., Zhu, X.J., et al. (2013). The contribution of TNF-α in the amygdala to anxiety in mice with persistent inflammatory pain. Neurosci. Lett. *541*: 275–280.

Chen, T., Koga, K., Descalzi, G., Qiu, S., Wang, J., Zhang, L.-S., et al. (2014). Postsynaptic potentiation of corticospinal projecting neurons in the anterior cingulate cortex after nerve injury. Mol. Pain *10*: 33.

Chen, Y., Mestek, A., Liu, J., and Yu, L. (1993). Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. Biochem. J. *295*: 625–628.

Cheng, Z.F., Fields, H.L., and Heinricher, M.M. (1986). Morphine microinjected into the periaqueductal gray has differential effects on 3 classes of medullary neurons. Brain Res. *375*: 57–65.

Cheriyan, J., and Sheets, P.L. (2018). Altered Excitability and Local Connectivity of mPFC-PAG Neurons in a Mouse Model of Neuropathic Pain. J. Neurosci. *38*: 4829–4839.

Chiara, G. Di, and Imperato, A. (1988). Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. J. Pharmacol. Exp. Ther. *244*: 1067–1080.

Chieng, B., and Christie, M.J. (1994). Inhibition by opioids acting on mu-receptors of GABAergic and glutamatergic postsynaptic potentials in single rat periaqueductal gray neurones in vitro. Br. J. Pharmacol. *113*: 303–309.

Chiou, C.-S., Chen, C.-C., Tsai, T.-C., Huang, C.-C., Chou, D., and Hsu, K.-S. (2016). Alleviating Bone Cancer-induced Mechanical Hypersensitivity by Inhibiting Neuronal Activity in the Anterior Cingulate Cortex. Anesthesiology *125*: 779–792.

Cho, H.J., and Basbaum, A.I. (1989). Arborization of single axons of the spinal dorsolateral funiculus to the contralateral superficial dorsal horn. Brain Res. *477*: 344–349.

Chrousos, G.P. (2009). Stress and disorders of the stress system. Nat. Rev. Endocrinol. *5*: 374–381.

Chung, E.K.Y., Bian, Z.-X., Xu, H.-X., and Sung, J.J.-Y. (2009). Neonatal maternal separation increases brain-derived neurotrophic factor and tyrosine kinase receptor B expression in the descending pain modulatory system. Neurosignals. *17*: 213–221.

Cleary, D.R., and Heinricher, M.M. (2013). Adaptations in responsiveness of brainstem pain-modulating neurons in acute compared with chronic inflammation. Pain *154*: 845–855.

Codd, E.E., Carson, J.R., Colburn, R.W., Stone, D.J., Besien, C.R. Van, Zhang, S.-P., et al. (2009). JNJ-20788560 [9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide], a selective delta opioid receptor agonist, is a potent and efficacious antihyperalgesic agent that does not produce respiratory depression, pharmacologic tole. J. Pharmacol. Exp. Ther. *329*: 241–251.

Coggeshall, R.E. (2005). Fos, nociception and the dorsal horn. Prog. Neurobiol. *77*: 299–352.

Commons, K.G. (2003). Translocation of presynaptic delta opioid receptors in the ventrolateral periaqueductal gray after swim stress. J. Comp. Neurol. *464*: 197–207.

Commons, K.G., Aicher, S.A., Kow, L.M., and Pfaff, D.W. (2000). Presynaptic and postsynaptic relations of mu-opioid receptors to gamma-aminobutyric acid-immunoreactive and medullary-projecting periaqueductal gray neurons. J. Comp. Neurol.

419: 532–542.

Commons, K.G., Bockstaele, E.J. van, and Pfaff, D.W. (1999). Frequent colocalization of mu opioid and NMDA-type glutamate receptors at postsynaptic sites in periaqueductal gray neurons. J. Comp. Neurol. *408*: 549–559.

Conti, F., Barbaresi, P., and Fabri, M. (1988). Cytochrome oxidase histochemistry reveals regional subdivisions in the rat periaqueductal gray matter. Neuroscience *24*: 629–633.

Cook, C.D., and Beardsley, P.M. (2003). The modulatory actions of dopamine D2/3 agonists and antagonists on the locomotor-activating effects of morphine and caffeine in mice. Pharmacol. Biochem. Behav. *75*: 363–371.

Corder, G., Castro, D.C., Bruchas, M.R., and Scherrer, G. (2018). Endogenous and Exogenous Opioids in Pain. Annu. Rev. Neurosci. *41*: 453–473.

Costa, B., Comelli, F., Bettoni, I., Colleoni, M., and Giagnoni, G. (2008). The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB(1), TRPV1 and PPARgamma receptors and neurotrophic factors. Pain *139*: 541–550.

Costa Gomez, T.M. Da, and Behbehani, M.M. (1995a). An electrophysiological characterization of the projection from the central nucleus of the amygdala to the periaqueductal gray of the rat: the role of opioid receptors. Brain Res. *689*: 21–31.

Costa Gomez, T.M. Da, and Behbehani, M.M. (1995b). An electrophysiological characterization of the projection from the central nucleus of the amygdala to the periaqueductal gray of the rat: the role of opioid receptors. Brain Res. *689*: 21–31.

Coutaux, A. (2017). Non-pharmacological treatments for pain relief: TENS and acupuncture. Jt. Bone Spine *84*: 657–661.

Crawley, J.N. (2004). Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment. Retard. Dev. Disabil. Res. Rev. *10*: 248–258.

Crettaz, B., Marziniak, M., Willeke, P., Young, P., Hellhammer, D., Stumpf, A., et al. (2013). Stress-induced allodynia--evidence of increased pain sensitivity in healthy humans and patients with chronic pain after experimentally induced psychosocial stress. PLoS One *8*: e69460.

Curatolo, M., Arendt-Nielsen, L., and Petersen-Felix, S. (2006). Central Hypersensitivity in Chronic Pain: Mechanisms and Clinical Implications. Phys. Med. Rehabil. Clin. N. Am. *17*: 287–302.

D'Souza, D., and Sadananda, M. (2017). Anxiety- and depressive-like profiles during early- and mid-adolescence in the female Wistar Kyoto rat. Int. J. Dev. Neurosci. *56*: 18–26.

Daly, R.J., Duggan, P.F., Bracken, P.J., Doonan, H.J., and Kelleher, N.J. (1987). Plasma levels of beta-endorphin in depressed patients with and without pain. Br. J. Psychiatry *150*: 224–7.

Dantas, G., Silva Torres, I.L. Da, Crema, L.M., Lara, D.R., and Dalmaz, C. (2005). Repeated restraint stress reduces opioid receptor binding in different rat CNS structures. Neurochem. Res. *30*: 1–7.

Daumas, S., Betourne, A., Halley, H., Wolfer, D.P., Lipp, H.P., Lassalle, J.M., et al. (2007). Transient activation of the CA3 Kappa opioid system in the dorsal hippocampus modulates complex memory processing in mice. Neurobiol. Learn. Mem. *88*: 94–103.

Davidson, R.J., and McEwen, B.S. (2012). Social influences on neuroplasticity: stress and interventions to promote well-being. Nat. Neurosci. *15*: 689–695.

Davis, G.C., Buchsbaum, M.S., Naber, D., Pickar, D., Post, R., Kammen, D. Van, et al. (1982). Altered Pain Perception and Cerebrospinal Endorphins in Psychiatric Illness. Ann. N. Y. Acad. Sci. *398*: 366–373.

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., et al. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature *405*: 183–187.

Deacon, R.M.J., and Rawlins, J.N.P. (2006). T-maze alternation in the rodent. Nat. Protoc. *1*: 7–12.

Deakin, J.F., and Dostrovsky, J.O. (1978). Involvement of the periaqueductal grey matter and spinal 5-hydroxytryptaminergic pathways in morphine analgesia: effcts of lesions and 5-hydroxytryptamine depletion. Br. J. Pharmacol. *63*: 159–165.

Delini-Stula, A., and Hunn, C. (1985). Neophobia in spontaneous hypertensive (SHR) and normotensive control (WKY) rats. Behav. Neural Biol. *43*: 206–211.

Dellarole, A., Morton, P., Brambilla, R., Walters, W., Summers, S., Bernardes, D., et al. (2014). Neuropathic pain-induced depressive-like behavior and hippocampal neurogenesis and plasticity are dependent on TNFR1 signaling. Brain. Behav. Immun. *41*: 65–81.

Dennis, T.S., Beck, K.D., Cominski, T.P., Bobzean, S.A.M., Kuzhikandathil, E. V., Servatius, R.J., et al. (2016). Exposure to morphine-associated cues increases mu opioid receptor mRNA expression in the nucleus accumbens of Wistar Kyoto rats. Behav. Brain Res. *313*: 208–213.

Depaoli, A.M., Hurley, K.M., Yasada, K., Refsine, T., and Bell, G. (1994). Distribution of κ opioid receptor mRNA in adult mouse brain: An in situ hybridization histochemistry study. Mol. Cell. Neurosci. *5*: 327–335.

Depaulis, A., Morgan, M.M., and Liebeskind, J.C. (1987). GABAergic modulation of the analgesic effects of morphine microinjected in the ventral periaqueductal gray matter of the rat. Brain Res. *436*: 223–228.

Derbyshire, S.W.G., Jones, A.K.P., Gyulai, F., Clark, S., Townsend, D., and Firestone, L.L. (1997). Pain processing during three levels of noxious stimulation produces differential patterns of central activity. Pain *73*: 431–445.

Descalzi, G., Mitsi, V., Purushothaman, I., Gaspari, S., Avrampou, K., Loh, Y.-H.E., et al. (2017). Neuropathic pain promotes adaptive changes in gene expression in brain networks involved in stress and depression. Sci. Signal. *10*: eaaj1549.

Deuis, J.R., Dvorakova, L.S., and Vetter, I. (2017). Methods Used to Evaluate Pain Behaviors in Rodents. Front. Mol. Neurosci. *10*: 284.

Devall, A.J., and Lovick, T.A. (2010). Differential activation of the periaqueductal gray by mild anxiogenic stress at different stages of the estrous cycle in female rats. Neuropsychopharmacology *35*: 1174–1185.

Devall, A.J., Santos, J.M., and Lovick, T.A. (2011). Estrous cycle stage influences on neuronal responsiveness to repeated anxiogenic stress in female rats. Behav. Brain Res. *225*: 334–340.

Dimitrov, E.L., Tsuda, M.C., Cameron, H.A., and Usdin, T.B. (2014). Anxiety- and

depression-like behavior and impaired neurogenesis evoked by peripheral neuropathy persist following resolution of prolonged tactile hypersensitivity. J. Neurosci. *34*: 12304–12312.

Dina, O.A., Khasar, S.G., Gear, R.W., and Levine, J.D. (2009). Activation of Gi induces mechanical hyperalgesia poststress or inflammation. Neuroscience *160*: 501–507.

Dixon, W.J. (1980). Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. *20*: 441–462.

Doharty, H. (2018). Characterisation of sex differences in behavioural domains and antidepressant response in two rat models of depression. PhD thesis. National University of Ireland Galway.

Drdla, R., Gassner, M., Gingl, E., and Sandkuhler, J. (2009). Induction of synaptic long-term potentiation after opioid withdrawal. Science *325*: 207–210.

Drolet, G., Proulx, K., Pearson, D., Rochford, J., and Deschepper, C.F. (2002). Comparisons of behavioral and neurochemical characteristics between WKY, WKHA, and Wistar rat strains. Neuropsychopharmacology *27*: 400–409.

Ducottet, C., and Belzung, C. (2004). Behaviour in the elevated plus-maze predicts coping after subchronic mild stress in mice. Physiol. Behav. *81*: 417–426.

Dufton, L.M., Konik, B., Colletti, R., Stanger, C., Boyer, M., Morrow, S., et al. (2008). Effects of stress on pain threshold and tolerance in children with recurrent abdominal pain. Pain *136*: 38–43.

Ehrich, E., Turncliff, R., Du, Y., Leigh-Pemberton, R., Fernandez, E., Jones, R., et al. (2015). Evaluation of opioid modulation in major depressive disorder. Neuropsychopharmacology *40*: 1448–1455.

Elsenbruch, S., Rosenberger, C., Bingel, U., Forsting, M., Schedlowski, M., and Gizewski, E.R. (2010). Patients with irritable bowel syndrome have altered emotional modulation of neural responses to visceral stimuli. Gastroenterology *139*: 1310-1319.e4.

Endres-Becker, J., Heppenstall, P.A., Mousa, S.A., Labuz, D., Oksche, A., Schafer, M., et al. (2007). Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. Mol. Pharmacol. *71*: 12–18.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav. Brain Res. *31*: 47–59.

Fahey, B., Barlow, S., Day, J.S., and O'Mara, S.M. (2008). Interferon-α-induced deficits in novel object recognition are rescued by chronic exercise. Physiol. Behav. *95*: 125–129.

Falcon, E., Maier, K., Robinson, S.A., Hill-Smith, T.E., and Lucki, I. (2015). Effects of buprenorphine on behavioral tests for antidepressant and anxiolytic drugs in mice. Psychopharmacology (Berl). *232*: 907–915.

Fang, F.G., Haws, C.M., Drasner, K., Williamson, A., and Fields, H.L. (1989a). Opioid peptides (DAGO-enkephalin, dynorphin A(1-13), BAM 22P) microinjected into the rat brainstem: comparison of their antinociceptive effect and their effect on neuronal firing in the rostral ventromedial medulla. Brain Res. *501*: 116–128.

Fang, F.G., Haws, C.M., Drasner, K., Williamson, A., and Fields, H.L. (1989b). Opioid peptides (DAGO-enkephalin, dynorphin A(1-13), BAM 22P) microinjected into the rat brainstem: comparison of their antinociceptive effect and their effect on neuronal firing

in the rostral ventromedial medulla. Brain Res. *501*: 116–128.

Fanselow, M.S., and Dong, H.W. (2010). Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures? Neuron *65*: 7–19.

Felice, M. De, Sanoja, R., Wang, R., Vera-Portocarrero, L., Oyarzo, J., King, T., et al. (2011). Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain. Pain *152*: 2701–2709.

Felice, V.D., Gibney, S.M., Gosselin, R.D., Dinan, T.G., O'Mahony, S.M., and Cryan, J.F. (2014). Differential activation of the prefrontal cortex and amygdala following psychological stress and colorectal distension in the maternally separated rat. Neuroscience *267*: 252–262.

Ferdousi, M., and Finn, D.P. (2018). Stress-induced modulation of pain: Role of the endogenous opioid system. Prog. Brain Res. *239*: 121–177.

Ferguson, S.A., and Cada, A.M. (2004). Spatial learning/memory and social and nonsocial behaviors in the Spontaneously Hypertensive, Wistar-Kyoto and Sprague-Dawley rat strains. Pharmacol. Biochem. Behav. *77*: 583–594.

Ferguson, S.A., Gray, E.P., and Cada, A.M. (2003). Early behavioral development in the spontaneously hypertensive rat: a comparison with the Wistar-Kyoto and Sprague-Dawley strains. Behav. Neurosci. *117*: 263–270.

Ferreira-Chamorro, P., Redondo, A., Riego, G., Leanez, S., and Pol, O. (2018). Sulforaphane Inhibited the Nociceptive Responses, Anxiety- and Depressive-Like Behaviors Associated With Neuropathic Pain and Improved the Anti-allodynic Effects of Morphine in Mice. Front. Pharmacol. *9*: 1332.

Ferreira, S.H., and Lorenzetti, B.B. (1994). Glutamate spinal retrograde sensitization of primary sensory neurons associated with nociception. Neuropharmacology *33*: 1479–1485.

Ferrini, F., Lorenzo, L.-E., Godin, A.G., Quang, M. Le, and Koninck, Y. De (2017). Enhancing KCC2 function counteracts morphine-induced hyperalgesia. Sci. Rep. *7*: 3870.

Ferrini, F., Trang, T., Mattioli, T.-A.M., Laffray, S., Del'Guidice, T., Lorenzo, L.-E., et al. (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl(-) homeostasis. Nat. Neurosci. *16*: 183–192.

Festing, M.F.W., and Altman, D.G. (2002). Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J. *43*: 244–258.

Fields, H.L., Anderson, S.D., Clanton, C.H., and Basbaum, A.I. (1976). Nucleus raphe magnus: a common mediator of opiate- and stimulus-produced analgesia. Trans. Am. Neurol. Assoc. *101*: 208–210.

Fields, H.L., Basbaum, A.I., Clanton, C.H., and Anderson, S.D. (1977). Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons. Brain Res. *126*: 441–453.

Fields, H.L., Bry, J., Hentall, I., and Zorman, G. (1983a). The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. J. Neurosci. *3*: 2545–2552.

Fields, H.L., Heinricher, M.M., and Mason, P. (1991). Neurotransmitters in nociceptive modulatory circuits. Annu. Rev. Neurosci. *14*: 219–245.

Fields, H.L., Malick, A., and Burstein, R. (1995). Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. J. Neurophysiol. *74*: 1742–1759.

Fields, H.L., Vanegas, H., Hentall, I.D., and Zorman, G. (1983b). Evidence that disinhibition of brain stem neurones contributes to morphine analgesia. Nature *306*: 684–686.

Fillingim, R.B., King, C.D., Ribeiro-Dasilva, M.C., Rahim-Williams, B., and Riley, J.L. (2009). Sex, Gender, and Pain: A Review of Recent Clinical and Experimental Findings. J. Pain *10*: 447–485.

Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H.W.D., Simonin, F., et al. (2000). Mice deficient for δ- and μ-opioid receptors exhibit opposing alterations of emotional responses. Nat. Genet. *25*: 195–200.

Finn, D.P., Jhaveri, M.D., Beckett, S.R.G., Roe, C.H., Kendall, D.A., Marsden, C.A., et al. (2003). Effects of direct periaqueductal grey administration of a cannabinoid receptor agonist on nociceptive and aversive responses in rats. Neuropharmacology *45*: 594–604.

Ford, G.K., and Finn, D.P. (2008). Clinical correlates of stress-induced analgesia: Evidence from pharmacological studies. Pain *140*: 3–7.

France, R.D., and Urban, B.J. (1991). Cerebrospinal fluid concentrations of beta-endorphin in chronic low back pain patients. Influence of depression and treatment. Psychosomatics *32*: 72–77.

France, R.D., Urban, B.J., Pelton, S., Kilts, C.D., Hong, J.S., and Nemeroff, C.B. (1987). CSF monoamine metabolites in chronic pain. Pain *31*: 189–198.

Fraser, G.L., Gaudreau, G.A., Clarke, P.B.S., Ménard, D.P., and Perkins, M.N. (2000). Antihyperalgesic effects of δ opioid agonists in a rat model of chronic inflammation. Br. J. Pharmacol. *129*: 1668–1672.

Fuchs, P.N., and McNabb, C.T. (2012). The place escape/avoidance paradigm: a novel method to assess nociceptive processing. J. Integr. Neurosci. *11*: 61–72.

Fukuda, K., Kato, S., Morikawa, H., Shoda, T., and Mori, K. (1996). Functional coupling of the delta-, mu-, and kappa-opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. J. Neurochem. *67*: 1309–1316.

Fukuhara, K., Ishikawa, K., Yasuda, S., Kishishita, Y., Kim, H.-K., Kakeda, T., et al. (2012). Intracerebroventricular 4-methylcatechol (4-MC) ameliorates chronic pain associated with depression-like behavior via induction of brain-derived neurotrophic factor (BDNF). Cell. Mol. Neurobiol. *32*: 971–977.

Gabbott, P.L.A., Warner, T.A., Jays, P.R.L., Salway, P., and Busby, S.J. (2005). Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. J. Comp. Neurol. *492*: 145–177.

Gadermann, A.M., Alonso, J., Vilagut, G., Zaslavsky, A.M., and Kessler, R.C. (2012). Comorbidity and disease burden in the National Comorbidity Survey Replication (NCS-R). Depress. Anxiety *29*: 797–806.

Gai, B.M., Bortolatto, C.F., Brüning, C.A., Zborowski, V.A., Stein, A.L., Zeni, G., et al. (2014). Depression-related behavior and mechanical allodynia are blocked by 3-(4-fluorophenylselenyl)-2,5-diphenylselenophene in a mouse model of neuropathic pain induced by partial sciatic nerve ligation. Neuropharmacology *79*: 580–589.

Gallantine, E.L., and Meer, T.F. (2005). A comparison of the antinociceptive and adverse

effects of the μ-opioid agonist morphine and the δ-opioid agonist SNC80. Basic Clin. Pharmacol. Toxicol. *97*: 39–51.

Gallantine, E.L., and Meert, T.F. (2008). Antinociceptive and adverse effects of mu- and kappa-opioid receptor agonists: a comparison of morphine and U50488-H. Basic Clin. Pharmacol. Toxicol. *103*: 419–427.

Gameiro, G.H., Gameiro, P.H., Silva Andrade, A. da, Pereira, L.F., Arthuri, M.T., Marcondes, F.K., et al. (2006). Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress. Physiol. Behav. *87*: 643–649.

Gameiro, G.H., Silva Andrade, A. Da, Castro, M. De, Pereira, L.F., Tambeli, C.H., and Ferraz De Arruda Veiga, M.C. (2005). The effects of restraint stress on nociceptive responses induced by formalin injected in rat's TMJ. Pharmacol. Biochem. Behav. *82*: 338–344.

Gannon, B., Finn, D.P., O'Gorman, D., Ruane, N., and McGuire, B.E. (2013). The cost of chronic pain: an analysis of a regional pain management service in Ireland. Pain Med. *14*: 1518–1528.

Gaskin, D.J., and Richard, P. (2012). The economic costs of pain in the United States. J. Pain *13*: 715–724.

Gauriau, C., and Bernard, J.-F. (2002). Pain pathways and parabrachial circuits in the rat. Exp. Physiol. *87*: 251–258.

Gavériaux-Ruff, C., Karchewski, L.A., Hever, X., Matifas, A., and Kieffer, B.L. (2008). Inflammatory pain is enhanced in delta opioid receptor-knockout mice. Eur. J. Neurosci. *27*: 2558–2567.

Gavériaux-Ruff, C., Nozaki, C., Nadal, X., Hever, X.C., Weibel, R., Matifas, A., et al. (2011). Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. Pain *152*: 1238–1248.

GBD 2017 Disease and Injury Incidence and Prevalence Collaborators (2018). Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet (London, England) *392*: 1789–1858.

Gentsch, C., Lichtsteiner, M., and Feer, H. (1987). Open field and elevated plus-maze: A behavioural comparison between spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats and the effects of chlordiazepoxide. Behav. Brain Res. *25*: 101–107.

Getachew, B., Hauser, S.R., Taylor, R.E., and Tizabi, Y. (2008). Desipramine blocks alcohol-induced anxiety- and depressive-like behaviors in two rat strains. Pharmacol. Biochem. Behav. *91*: 97–103.

Gibney, S.M., Gosselin, R.D., Dinan, T.G., and Cryan, J.F. (2010). Colorectal distension-induced prefrontal cortex activation in the Wistar-Kyoto rat: implications for irritable bowel syndrome. Neuroscience *165*: 675–683.

Gillett, K., Harshberger, E., and Valdez, G.R. (2013). Protracted withdrawal from ethanol and enhanced responsiveness stress: Regulation via the dynorphin/kappa opioid receptor system. Alcohol *47*: 359–365.

Girardot, M.N., and Holloway, F.A. (1984). Intermittent cold water stress-analgesia in rats: cross-tolerance to morphine. Pharmacol. Biochem. Behav. *20*: 631–633.

Glover, E.M., and Davis, M. (2008). Anxiolytic-like effects of morphine and buprenorphine in the rat model of fear-potentiated startle: tolerance, cross-tolerance, and blockade by naloxone. Psychopharmacology (Berl). *198*: 167–180.

Goffer, Y., Xu, D., Eberle, S.E., D'amour, J., Lee, M., Tukey, D., et al. (2013). Calcium-permeable AMPA receptors in the nucleus accumbens regulate depression-like behaviors in the chronic neuropathic pain state. J. Neurosci. *33*: 19034–19044.

Gogas, K.R., Cho, H.J., Botchkina, G.I., Levine, J.D., and Basbaum, A.I. (1996). Inhibition of noxious stimulus-evoked pain behaviors and neuronal fos-like immunoreactivity in the spinal cord of the rat by supraspinal morphine. Pain *65*: 9–15.

Gogas, K.R., Presley, R.W., Levine, J.D., and Basbaum, A.I. (1991). The antinociceptive action of supraspinal opioids results from an increase in descending inhibitory control: Correlation of nociceptive behavior and c-fos expression. Neuroscience *42*: 617–628.

Gonçalves, L., Silva, R., Pinto-Ribeiro, F., Pêgo, J.M., Bessa, J.M., Pertovaara, A., et al. (2008). Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. Exp. Neurol. *213*: 48–56.

Gonzales, E.L.T., Yang, S.M., Choi, C.S., Mabunga, D.F.N., Kim, H.J., Cheong, J.H., et al. (2015). Repeated neonatal propofol administration induces sex-dependent long-term impairments on spatial and recognition memory in rats. Biomol. Ther. *23*: 251–260.

Gonzalez-Sepulveda, M., Pozo, O.J., Marcos, J., and Valverde, O. (2016). Chronic pain causes a persistent anxiety state leading to increased ethanol intake in CD1 mice. J. Psychopharmacol. *30*: 188–203.

González, D., Riba, J., Bouso, J.C., Gómez-Jarabo, G., and Barbanoj, M.J. (2006). Pattern of use and subjective effects of Salvia divinorum among recreational users. Drug Alcohol Depend. *85*: 157–162.

Grace, P.M., Maier, S.F., and Watkins, L.R. (2015). Opioid-induced central immune signaling: implications for opioid analgesia. Headache *55*: 475–489.

Grande, L.A., Loeser, J.D., Ozuna, J., Ashleigh, A., and Samii, A. (2004). Complex regional pain syndrome as a stress response. Pain *110*: 495–498.

Granier, S., Manglik, A., Kruse, A.C., Kobilka, T.S., Thian, F.S., Weis, W.I., et al. (2012). Structure of the delta-opioid receptor bound to naltrindole. Nature *485*: 400–404.

Grauer, E., and Kapon, Y. (1993). Wistar-Kyoto rats in the Morris water maze: Impaired working memory and hyper-reactivity to stress. Behav. Brain Res. *59*: 147–151.

Green, P.G., Chen, X., Alvarez, P., Ferrari, L.F., and Levine, J.D. (2011). Early-life stress produces muscle hyperalgesia and nociceptor sensitization in the adult rat. Pain *152*: 2549–2556.

Greenwood-Van Meerveld, B., Johnson, A.C., Cochrane, S., Schulkin, J., and Myers, D.A. (2005). Corticotropin-releasing factor 1 receptor-mediated mechanisms inhibit colonic hypersensitivity in rats. Neurogastroenterol. Motil. *17*: 415–422.

Gregoire, S., Michaud, V., Chapuy, E., Eschalier, A., and Ardid, D. (2012). Study of emotional and cognitive impairments in mononeuropathic rats: effect of duloxetine and gabapentin. Pain *153*: 1657–1663.

Grégoire, S., Wattiez, A.S., Etienne, M., Marchand, F., and Ardid, D. (2014). Monoarthritis-induced emotional and cognitive impairments in rats are sensitive to low systemic doses or intra-amygdala injections of morphine. Eur. J. Pharmacol. *735*: 1–9.

Grimwood, S., Lu, Y., Schmidt, A.W., Vanase-Frawley, M.A., Sawant-Basak, A., Miller, E., et al. (2011). Pharmacological characterization of 2-methyl-N-((2'-(pyrrolidin-1-ylsulfonyl)biphenyl-4-yl)methyl)propan-1-amine (PF-04455242), a high-affinity antagonist selective for kappa-opioid receptors. J. Pharmacol. Exp. Ther. *339*: 555–566.

Grover, S., Kumar, V., Chakrabarti, S., Hollikatti, P., Singh, P., Tyagi, S., et al. (2012). Prevalence and type of functional somatic complaints in patients with first-episode depression. East Asian Arch. Psychiatry Off. J. Hong Kong Coll. Psychiatr. = Dong Ya Jing Shen Ke Xue Zhi Xianggang Jing Shen Ke Yi Xue Yuan Qi Kan *22*: 146–153.

Guerrero-Alba, R., Valdez Morales, E.E., Jimenez-Vargas, J.N., Lopez, C.L., Polanco, J.J., Okamoto, T., et al. (2017). Stress activates pronociceptive endogenous opioid signalling in DRG neurons during chronic colitis. Gut *66*: 2121-2131.

Gunter, W.D., Myers, D.A., Beverley, Foreman, R.D., and Shepard, J.D. (2002). Evidence for visceral hypersensitivity in high-anxiety rats. Physiol. Behav. *69*: 379–382.

Guo, H. liang, Xiao, Y., Tian, Z., Li, X. bo, Wang, D. sheng, Wang, X. shang, et al. (2016). Anxiolytic effects of sesamin in mice with chronic inflammatory pain. Nutr. Neurosci. *19*: 231–236.

Gutstein, H.B., Mansour, A., Watson, S.J., Akil, H., and Fields, H.L. (1998). Mu and kappa opioid receptors in periaqueductal gray and rostral ventromedial medulla. Neuroreport *9*: 1777–1781.

Hang, A., Wang, Y., He, L., and Liu, J. (2015). The role of the dynorphin/kappa opioid receptor system in anxiety. Acta Pharmacol. Sin. *36*: 783–790.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain *32*: 77–88.

Hargreaves, K.M., Dionne, R.A., and Mueller, G.P. (1983). Plasma Beta-Endorphin-like Immunoreactivity, Pain and Anxiety Following Administration of Placebo in Oral Surgery Patients. J. Dent. Res. *62*: 1170–1173.

Harris, J.A. (1998). Using c-fos as a neural marker of pain. Brain Res. Bull. *45*: 1–8.

Harris, R.E., Clauw, D.J., Scott, D.J., McLean, S.A., Gracely, R.H., and Zubieta, J.-K. (2007). Decreased Central -Opioid Receptor Availability in Fibromyalgia. J. Neurosci. *27*: 10000–10006.

Hashmi, J.A., Baliki, M.N., Huang, L., Baria, A.T., Torbey, S., Hermann, K.M., et al. (2013). Shape shifting pain: chronification of back pain shifts brain representation from nociceptive to emotional circuits. Brain *136*: 2751–2768.

Hasnie, F.S., Wallace, V.C.J., Hefner, K., Holmes, A., and Rice, A.S.C. (2007). Mechanical and cold hypersensitivity in nerve-injured C57BL/6J mice is not associated with fear-avoidance- and depression-related behaviour. Br. J. Anaesth. *98*: 816–822.

Hawker, G.A., Gignac, M.A.M., Badley, E., Davis, A.M., French, M.R., Li, Y., et al. (2011). A longitudinal study to explain the pain-depression link in older adults with osteoarthritis. Arthritis Care Res. (Hoboken). *63*: 1382–1390.

Hayes, A.G., Sheehan, M.J., and Tyers, M.B. (1987). Differential sensitivity of models of antinociception in the rat, mouse and guinea-pig to mu- and kappa-opioid receptor agonists. Br. J. Pharmacol. *91*: 823–832.

Hebb, A.L.O., Poulin, J.F., Roach, S.P., Zacharko, R.M., and Drolet, G. (2005).

Cholecystokinin and endogenous opioid peptides: Interactive influence on pain, cognition, and emotion. Prog. Neuro-Psychopharmacology Biol. Psychiatry *29*: 1225–1238.

Heer, E.W. de, Gerrits, M.M.J.G., Beekman, A.T.F., Dekker, J., Marwijk, H.W.J. van, Waal, M.W.M. de, et al. (2014). The association of depression and anxiety with pain: a study from NESDA. PLoS One *9*: e106907.

Heidari, J., Mierswa, T., Kleinert, J., Ott, I., Levenig, C., Hasenbring, M., et al. (2016). Parameters of low back pain chronicity among athletes: Associations with physical and mental stress. Phys. Ther. Sport *21*: 31–37.

Heinricher, M.M., Barbaro, N.M., and Fields, H.L. (1989). Putative nociceptive modulating neurons in the rostral ventromedial medulla of the rat: firing of on- and off-cells is related to nociceptive responsiveness. Somatosens. Mot. Res. *6*: 427–439.

Heinricher, M.M., Morgan, M.M., and Fields, H.L. (1992a). Direct and indirect actions of morphine on medullary neurons that modulate nociception. Neuroscience *48*: 533–543.

Heinricher, M.M., Morgan, M.M., and Fields, H.L. (1992b). Direct and indirect actions of morphine on medullary neurons that modulate nociception. Neuroscience *48*: 533–543.

Heinricher, M.M., Morgan, M.M., Tortorici, V., and Fields, H.L. (1994). Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. Neuroscience *63*: 279–288.

Heinricher, M.M., Tavares, I., Leith, J.L., and Lumb, B.M. (2009). Descending control of nociception: Specificity, recruitment and plasticity. Brain Res. Rev. *60*: 214–225.

Helmstetter, F.J., Tershner, S.A., Poore, L.H., and Bellgowan, P.S.. (1998). Antinociception following opioid stimulation of the basolateral amygdala is expressed through the periaqueductal gray and rostral ventromedial medulla. Brain Res. *779*: 104–118.

Henry, M.S., Bisht, K., Vernoux, N., Gendron, L., Torres-Berrio, A., Drolet, G., et al. (2018). Delta Opioid Receptor Signaling Promotes Resilience to Stress Under the Repeated Social Defeat Paradigm in Mice. Front. Mol. Neurosci. *11*: 100.

Hernandez-Leon, A., Fernandez-Guasti, A., and Gonzalez-Trujano, M.E. (2016). Rutin antinociception involves opioidergic mechanism and descending modulation of ventrolateral periaqueductal grey matter in rats. Eur. J. Pain *20*: 274–283.

Hersh, L.B. (1985). Characterization of membrane-bound aminopeptidases from rat brain: identification of the enkephalin-degrading aminopeptidase. J. Neurochem. *44*: 1427–1435.

Hestehave, S., Abelson, K.S., Brønnum Pedersen, T., and Munro, G. (2019a). Stress sensitivity and cutaneous sensory thresholds before and after neuropathic injury in various inbred and outbred rat strains. Behav. Brain Res. *375*: 112149.

Hestehave, S., Abelson, K.S.P., Brønnum Pedersen, T., and Munro, G. (2019b). The analgesic efficacy of morphine varies with rat strain and experimental pain model: implications for target validation efforts in pain drug discovery. Eur. J. Pain *23*: 539–554.

Ho, I.K., Loh, H.H., and Way, E.L. (1973). Cyclic adenosine monophosphate antagonism of morphine analgesia. J. Pharmacol. Exp. Ther. *185*: 336–346.

Hodge, C.J.J., and Apkarian, A. V (1990). The spinothalamic tract. Crit. Rev. Neurobiol. *5*: 363–397.

Hoffmann, O., Plesan, A., and Wiesenfeld-Hallin, Z. (1998). Genetic differences in morphine sensitivity, tolerance and withdrawal in rats. Brain Res. *806*: 232–237.

Hokfelt, T., Kellerth, J.O., Nilsson, G., and Pernow, B. (1975). Substance p: localization in the central nervous system and in some primary sensory neurons. Science *190*: 889–890.

Holden, J.E., Jeong, Y., and Forrest, J.M. (2005). The endogenous opioid system and clinical pain management. AACN Clin. Issues *16*: 291–301.

Holdridge, S. V, and Cahill, C.M. (2007). Spinal administration of a delta opioid receptor agonist attenuates hyperalgesia and allodynia in a rat model of neuropathic pain. Eur. J. Pain *11*: 685–693.

Holschneider, D.P., Guo, Y., Mayer, E.A., and Wang, Z. (2016). Early life stress elicits visceral hyperalgesia and functional reorganization of pain circuits in adult rats. Neurobiol. Stress *3*: 8–22.

Hopkins, D.A., and Holstege, G. (1978). Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. Exp. Brain Res. *32*: 529–547.

Hormozi, A., Zarifkar, A., Rostami, B., and Naghibalhossaini, F. (2019). An Experimental Study on Spinal Cord micro-Opioid and alpha2-Adrenergic Receptors mRNA Expression Following Stress-Induced Hyperalgesia in Male Rats. Iran. J. Med. Sci. *44*: 397–405.

Hormozi, A., Zarifkar, A., Tatar, M., Barazesh, M., and Rostami, B. (2018). Effects of Post-Weaning Chronic Stress on Nociception, Spinal Cord mu-Opioid, and alpha2-Adrenergic Receptors Expression in Rats and Their Offspring. J. Mol. Neurosci. *64*: 567–573.

Hu, B., Doods, H., Treede, R.-D., and Ceci, A. (2009). Depression-like behaviour in rats with mononeuropathy is reduced by the CB2-selective agonist GW405833. Pain *143*: 206–212.

Hu, C., Luo, Y., Wang, H., Kuang, S., Liang, G., Yang, Y., et al. (2017). Re-evaluation of the interrelationships among the behavioral tests in rats exposed to chronic unpredictable mild stress. PLoS One *12*: e0185129.

Hu, Y., Yang, J., Hu, Y., Wang, Y., and Li, W. (2010). Amitriptyline rather than lornoxicam ameliorates neuropathic pain-induced deficits in abilities of spatial learning and memory. Eur. J. Anaesthesiol. *27*: 162–168.

Huang, J., Wang, Y., Wang, W., Wei, Y., Li, Y., and Wu, S. (2008). Preproenkephalin mRNA is expressed in a subpopulation of GABAergic neurons in the spinal dorsal horn of the GAD67-GFP knock-in mouse. Anat. Rec. (Hoboken). *291*: 1334–1341.

Huang, K.H., and Shyu, B.C. (1987). Differential stress effects on responses to noxious stimuli as measured by tail-flick latency and squeak threshold in rats. Acta Physiol. Scand. *129*: 401–406.

Huang, N., Yang, C., Hua, D., Li, S., Zhan, G., Yang, N., et al. (2019). Alterations in the BDNF–mTOR Signaling Pathway in the Spinal Cord Contribute to Hyperalgesia in a Rodent Model of Chronic Restraint Stress. Neuroscience *409*: 142–151.

Huang, P., Tunis, J., Parry, C., Tallarida, R., and Liu-Chen, L.Y. (2016). Synergistic antidepressant-like effects between a kappa opioid antagonist (LY2444296) and a delta opioid agonist (ADL5859) in the mouse forced swim test. Eur. J. Pharmacol. *781*: 53–59.

Hudson, P.M., and Lumb, B.M. (1996). Neurones in the midbrain periaqueductal grey send collateral projections to nucleus raphe magnus and the rostral ventrolateral medulla in the rat. Brain Res. *733*: 138–141.

Hughes, J. (1975). Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. Brain Res. *88*: 295–308.

Hunt, S.P., Pini, A., and Evan, G. (1987). Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature *328*: 632–634.

Hurley, R.W., and Hammond, D.L. (2000). The analgesic effects of supraspinal mu and delta opioid receptor agonists are potentiated during persistent inflammation. J. Neurosci. *20*: 1249–1259.

Hyland, N.P., O'Mahony, S.M., O'Malley, D., O'Mahony, C.M., Dinan, T.G., and Cryan, J.F. (2015). Early-life stress selectively affects gastrointestinal but not behavioral responses in a genetic model of brain-gut axis dysfunction. Neurogastroenterol. Motil. *27*: 105–113.

Hylands-White, N., Duarte, R. V., and Raphael, J.H. (2017). An overview of treatment approaches for chronic pain management. Rheumatol. Int. *37*: 29–42.

IASP Task Force on Taxonomy (1994). Chronic pain classification; Pain-terminology. In Classification of Chronic Pain., H. Merskey, and N. Bogduk, eds. (Seattle: IASP Press), pp 209–214.

Ide, S., Satoyoshi, H., Minami, M., and Satoh, M. (2015). Amelioration of the reduced antinociceptive effect of morphine in the unpredictable chronic mild stress model mice by noradrenalin but not serotonin reuptake inhibitors. Mol. Pain *11*: 47.

Ikoma, M., Kohno, T., and Baba, H. (2007). Differential presynaptic effects of opioid agonists on Adelta- and C-afferent glutamatergic transmission to the spinal dorsal horn. Anesthesiology *107*: 807–812.

Imbe, H., and Kimura, A. (2018). Increase of histone acetylation in the GABAergic neurons in the rostral ventromedial medulla associated with mechanical hypersensitivity after repeated restraint stress. Brain Res. Bull. *142*: 394–402.

Imbe, H., Kimura, A., Donishi, T., and Kaneoke, Y. (2012). Chronic restraint stress decreases glial fibrillary acidic protein and glutamate transporter in the periaqueductal gray matter. Neuroscience *223*: 209–218.

Imbe, H., Kimura, A., Donishi, T., and Kaneoke, Y. (2013). Effects of restraint stress on glial activity in the rostral ventromedial medulla. Neuroscience *241*: 10–21.

Imbe, H., Murakami, S., Okamoto, K., Iwai-Liao, Y., and Senba, E. (2004). The effects of acute and chronic restraint stress on activation of ERK in the rostral ventromedial medulla and locus coeruleus. Pain *112*: 361–371.

Imbe, H., Okamoto, K., Donishi, T., Senba, E., and Kimura, A. (2010). Involvement of descending facilitation from the rostral ventromedial medulla in the enhancement of formalin-evoked nocifensive behavior following repeated forced swim stress. Brain Res. *1329*: 103–112.

Jackson, A., and Cooper, S.J. (1988). Observational analysis of the effects of kappa opioid agonists an open field behaviour in the rat. Psychopharmacology (Berl). *94*: 248–253.

Jacobson, M.L., Wulf, H.A., Browne, C.A., and Lucki, I. (2018). Opioid modulation of cognitive impairment in depression. In Progress in Brain Research *239*: 1–48.

Jacquet, Y.F., and Lajtha, A. (1974). Paradoxical effects after microinjection of morphine in the periaqueductal gray matter in the rat. Science *185*: 1055–1057.

Jahr, C.E., and Jessell, T.M. (1983). ATP excites a subpopulation of rat dorsal horn neurones. Nature *304*: 730–733.

Jain, P., Hassan, A.M., Koyani, C.N., Mayerhofer, R., Reichmann, F., Farzi, A., et al. (2015). Behavioral and molecular processing of visceral pain in the brain of mice: impact of colitis and psychological stress. Front. Behav. Neurosci. *9*: 177.

Jamison, R.N., Edwards, R.R., Liu, X., Ross, E.L., Michna, E., Warnick, M., et al. (2013). Relationship of negative affect and outcome of an opioid therapy trial among low back pain patients. Pain Pract. *13*: 173–181.

Jamot, L., Matthes, H.W.D., Simonin, F., Kieffer, B.L., and Roder, J.C. (2003). Differential involvement of the mu and kappa opioid receptors in spatial learning. Genes. Brain. Behav. *2*: 80–92.

Jang, C.G., Lee, S.Y., Yoo, J.H., Yan, J.J., Song, D.K., Loh, H.H., et al. (2003). Impaired water maze learning performance in μ-opioid receptor knockout mice. Mol. Brain Res. *117*: 68–72.

Jennings, E.M. (2015). The role of the endocannabinoid system in the affective modulation of pain. PhD thesis. National University of Ireland Galway.

Jennings, E.M., Okine, B.N., Olango, W.M., Roche, M., and Finn, D.P. (2016). Repeated forced swim stress differentially affects formalin-evoked nociceptive behaviour and the endocannabinoid system in stress normo-responsive and stress hyper-responsive rat strains. Prog. Neuro-Psychopharmacology Biol. Psychiatry *64*: 181–189.

Jennings, E.M., Okine, B.N., Roche, M., and Finn, D.P. (2014). Stress-induced hyperalgesia. Prog. Neurobiol. *121*: 1–18.

Jensen, T.S., and Yaksh, T.L. (1986). III. Comparison of the antinociceptive action of Mu and delta opioid receptor ligands in the periaqueductal gray matter, medial and paramedial ventral medulla in the rat as studied by the microinjection technique. Brain Res. *372*: 301–312.

Jesse, C.R., Wilhelm, E.A., and Nogueira, C.W. (2010). Depression-like behavior and mechanical allodynia are reduced by bis selenide treatment in mice with chronic constriction injury: a comparison with fluoxetine, amitriptyline, and bupropion. Psychopharmacology (Berl). *212*: 513–522.

Ji, G., Fu, Y., Ruppert, K.A., and Neugebauer, V. (2007). Pain-related anxiety-like behavior requires CRF1 receptors in the amygdala. Mol. Pain *3*: 13.

Ji, G., Zhang, W., Mahimainathan, L., Narasimhan, M., Kiritoshi, T., Fan, X., et al. (2017). 5-HT2C Receptor Knockdown in the Amygdala Inhibits Neuropathic-Pain-Related Plasticity and Behaviors. J. Neurosci. *37*: 1378–1393.

Jiang, H., Fang, D., Kong, L.-Y., Jin, Z.-R., Cai, J., Kang, X.-J., et al. (2014a). Sensitization of neurons in the central nucleus of the amygdala via the decreased GABAergic inhibition contributes to the development of neuropathic pain-related anxiety-like behaviors in rats. Mol. Brain *7*: 72.

Jiang, Z.-C., Qi, W.-J., Wang, J.-Y., and Luo, F. (2014b). Chronic administration of 5-HT1A receptor agonist relieves depression and depression-induced hypoalgesia. ScientificWorldJournal. *2014*: 405736.

Johansen, J.P., Fields, H.L., and Manning, B.H. (2001). The affective component of pain in rodents: Direct evidence for a contribution of the anterior cingulate cortex. Proc. Natl. Acad. Sci. *98*: 8077–8082.

Johnson, A.C., Tran, L., Schulkin, J., and Greenwood-Van Meerveld, B. (2012). Importance of stress receptor-mediated mechanisms in the amygdala on visceral pain perception in an intrinsically anxious rat. Neurogastroenterol. Motil. *24*: 479–86, e219.

Jones, A.K.P., Kitchen, N.D., Watabe, H., Cunningham, V.J., Jones, T., Luthra, S.K., et al. (1999). Measurement of Changes in Opioid Receptor Binding in Vivo During Trigeminal Neuralgic Pain Using [11C]Diprenorphine and Positron Emission Tomography. J. Cereb. Blood Flow Metab. *19*: 803–808.

Julius, D. (2013). TRP channels and pain. Annu. Rev. Cell Dev. Biol. *29*: 355–384.

Jutkiewicz, E.M., Eller, E.B., Folk, J.E., Rice, K.C., Traynor, J.R., and Woods, J.H. (2004). Delta-opioid agonists: differential efficacy and potency of SNC80, its 3-OH (SNC86) and 3-desoxy (SNC162) derivatives in Sprague-Dawley rats. J. Pharmacol. Exp. Ther. *309*: 173–181.

Jutkiewicz, E.M., Torregrossa, M.M., Sobczyk-Kojiro, K., Mosberg, H.I., Folk, J.E., Rice, K.C., et al. (2006). Behavioral and neurobiological effects of the enkephalinase inhibitor RB101 relative to its antidepressant effects. Eur. J. Pharmacol. *531*: 151–159.

Kabli, N., and Cahill, C.M. (2007). Anti-allodynic effects of peripheral delta opioid receptors in neuropathic pain. Pain *127*: 84–93.

Kain, Z.N., Sevarino, F., Alexander, G.M., Pincus, S., and Mayes, L.C. (2000). Preoperative anxiety and postoperative pain in women undergoing hysterectomy. A repeated-measures design. J. Psychosom. Res. *49*: 417–422.

Kalinichev, M., Easterling, K.W., and Holtzman, S.G. (2001). Repeated neonatal maternal separation alters morphine-induced antinociception in male rats. Brain Res. Bull. *54*: 649–654.

Kalueff, A. V, Stewart, A.M., Song, C., Berridge, K.C., Graybiel, A.M., and Fentress, J.C. (2016). Neurobiology of rodent self-grooming and its value for translational neuroscience. Nat. Rev. Neurosci. *17*: 45–59.

Kang, S.J., Kwak, C., Lee, J., Sim, S.-E., Shim, J., Choi, T., et al. (2015). Bidirectional modulation of hyperalgesia via the specific control of excitatory and inhibitory neuronal activity in the ACC. Mol. Brain *8*: 81.

Kapitzke, D., Vetter, I., and Cabot, P.J. (2005). Endogenous opioid analgesia in peripheral tissues and the clinical implications for pain control. Ther. Clin. Risk Manag. *1*: 279–297.

Kastenberger, I., Lutsch, C., Herzog, H., and Schwarzer, C. (2012). Influence of sex and genetic background on anxiety-related and stress-induced behaviour of prodynorphin-deficient mice. PLoS One *7*: e34251.

Keay, K.A., and Bandler, R. (1993). Deep and superficial noxious stimulation increases Fos-like immunoreactivity in different regions of the midbrain periaqueductal grey of the rat. Neurosci. Lett. *154*: 23–26.

Keay, K.A., and Bandler, R. (2001). Parallel circuits mediating distinct emotional coping reactions to different types of stress. Neurosci. Biobehav. Rev. *25*: 669–678.

Keay, K.A., and Bandler, R. (2002). Distinct central representations of inescapable and escapable pain: observations and speculation. Exp. Physiol. *87*: 275–279.

Keller, A.F., Beggs, S., Salter, M.W., and Koninck, Y. De (2007). Transformation of the output of spinal lamina I neurons after nerve injury and microglia stimulation underlying neuropathic pain. Mol. Pain *3*: 27.

Khasar, S.G., Burkham, J., Dina, O.A., Brown, A.S., Bogen, O., Alessandri-Haber, N., et al. (2008). Stress induces a switch of intracellular signaling in sensory neurons in a model of generalized pain. J. Neurosci. *28*: 5721–5730.

Kieffer, B.L., Befort, K., Gaveriaux-Ruff, C., and Hirth, C.G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc. Natl. Acad. Sci. U. S. A. *89*: 12048–12052.

Kiguchi, N., Ding, H., and Ko, M.C. (2016). Central N/OFQ-NOP Receptor System in Pain Modulation. In Advances in Pharmacology, (Academic Press), pp 217–243.

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., and Altman, D.G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. *8*: e1000412.

Kim, H.-Y. (2015). Statistical notes for clinical researchers: Type I and type II errors in statistical decision. Restor. Dent. Endod. *40*: 249–252.

Kim, H., Chen, L., Lim, G., Sung, B., Wang, S., McCabe, M.F., et al. (2012). Brain indoleamine 2,3-dioxygenase contributes to the comorbidity of pain and depression. J. Clin. Invest. *122*: 2940–2954.

Kim, K.W., Choi, S.S., Woo, R.S., and Suh, H.W. (2003). Development of antinociceptive tolerance and changes of opioid receptor ligand binding in central nervous system of the mouse forced to single and repeated swimming in the cold water. Brain Res. Bull. *61*: 93–97.

Kim, S.-H., Moon, I.S., and Park, I.-S. (2013). Unique hippocampal changes and allodynia in a model of chronic stress. J. Korean Med. Sci. *28*: 946–950.

Kincaid, W., Neubert, M.J., Xu, M., Kim, C.J., and Heinricher, M.M. (2006). Role for medullary pain facilitating neurons in secondary thermal hyperalgesia. J. Neurophysiol. *95*: 33–41.

Kindler, L.L., Sibille, K.T., Glover, T.L., Staud, R., Riley, J.L., and Fillingim, R.B. (2011). Drug Response Profiles to Experimental Pain Are Opioid and Pain Modality Specific. J. Pain *12*: 340–351.

Kiritoshi, T., and Neugebauer, V. (2015). Group II mGluRs modulate baseline and arthritis pain-related synaptic transmission in the rat medial prefrontal cortex. Neuropharmacology *95*: 388–394.

Kivell, B., and Prisinzano, T.E. (2010). Kappa opioids and the modulation of pain. Psychopharmacology (Berl). *210*: 109–119.

Klega, A., Eberle, T., Buchholz, H.G., Maus, S., Maihöfner, C., Schreckenberger, M., et al. (2010). Central opioidergic neurotransmission in complex regional pain syndrome. Neurology *75*: 129–136.

Klein, T., Magerl, W., Nickel, U., Hopf, H.C., Sandkühler, J., and Treede, R.D. (2007). Effects of the NMDA-receptor antagonist ketamine on perceptual correlates of long-term potentiation within the nociceptive system. Neuropharmacology *52*: 655–661.

Knapp, R.J., Malatynska, E., Fang, L., Li, X., Babin, E., Nguyen, M., et al. (1994). Identification of a human delta opioid receptor: cloning and expression. Life Sci. *54*:

PL463-9.

Knaster, P., Karlsson, H., Estlander, A.M., and Kalso, E. (2012). Psychiatric disorders as assessed with SCID in chronic pain patients: The anxiety disorders precede the onset of pain. Gen. Hosp. Psychiatry *34*: 46–52.

Knoll, A.T., and Carlezon, W.A. (2010). Dynorphin, stress, and depression. Brain Res. *1314*: 56–73.

Knoll, A.T., Meloni, E.G., Thomas, J.B., Carroll, F.I., and Carlezon, W.A.J. (2007). Anxiolytic-like effects of kappa-opioid receptor antagonists in models of unlearned and learned fear in rats. J. Pharmacol. Exp. Ther. *323*: 838–845.

Knoll, A.T., Muschamp, J.W., Sillivan, S.E., Ferguson, D., Dietz, D.M., Meloni, E.G., et al. (2011). Kappa opioid receptor signaling in the basolateral amygdala regulates conditioned fear and anxiety in rats. Biol. Psychiatry *70*: 425–433.

Kodama, D., Ono, H., and Tanabe, M. (2011). Increased hippocampal glycine uptake and cognitive dysfunction after peripheral nerve injury. Pain *152*: 809–817.

Kõks, S., Soosaar, A., Võikar, V., Bourin, M., and Vasar, E. (1999). BOC-CCK-4, CCK(B) receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. Neuropeptides *33*: 63–69.

Koltzenburg, M., Pokorny, R., Gasser, U.E., and Richarz, U. (2006). Differential sensitivity of three experimental pain models in detecting the analgesic effects of transdermal fentanyl and buprenorphine. Pain *126*: 165–174.

Kondo, I., Marvizon, J.C.G., Song, B., Salgado, F., Codeluppi, S., Hua, X.-Y., et al. (2005). Inhibition by spinal mu- and delta-opioid agonists of afferent-evoked substance P release. J. Neurosci. *25*: 3651–3660.

Kontinen, V.K., Kauppila, T., Paananen, S., Pertovaara, A., and Kalso, E. (1999). Behavioural measures of depression and anxiety in rats with spinal nerve ligation-induced neuropathy. Pain *80*: 341–346.

Kosterlitz, H.W. (1985). The Wellcome Foundation lecture, 1982. Opioid peptides and their receptors. Proc. R. Soc. London. Ser. B, Biol. Sci. *225*: 27–40.

Kraatz, S., Lang, J., Kraus, T., Munster, E., and Ochsmann, E. (2013). The incremental effect of psychosocial workplace factors on the development of neck and shoulder disorders: a systematic review of longitudinal studies. Int. Arch. Occup. Environ. Health *86*: 375–395.

Kreibich, A., Reyes, B.A.S., Curtis, A.L., Ecke, L., Chavkin, C., Bockstaele, E.J. Van, et al. (2008). Presynaptic inhibition of diverse afferents to the locus ceruleus by kappa-opiate receptors: a novel mechanism for regulating the central norepinephrine system. J. Neurosci. *28*: 6516–6525.

Kroenke, K., Wu, J., Bair, M.J., Krebs, E.E., Damush, T.M., and Tu, W. (2011). Reciprocal relationship between pain and depression: A 12-month longitudinal analysis in primary care. J. Pain *12*: 964–973.

Kudryavtseva, N.N., Gerrits, M.A., Alekseenko, O. V, and Ree, J.M. Van (2005). Chronic cocaine injections attenuate behavioral response of kappa-opioid receptors to U-50,488H agonist. Bull. Exp. Biol. Med. *140*: 320–322.

Kuehl, L.K., Michaux, G.P., Richter, S., Schächinger, H., and Anton, F. (2010). Increased basal mechanical pain sensitivity but decreased perceptual wind-up in a human model of

relative hypocortisolism. Pain *149*: 539–546.

Kunihara, M., Ohyama, M., and Nakano, M. (1993). Effects of spiradoline mesylate, a selective kappa-opioid-receptor agonist, on the central dopamine system with relation to mouse locomotor activity and analgesia. Jpn. J. Pharmacol. *62*: 223–230.

Kurtz, T.W., Montano, M., Chan, L., and Kabra, P. (1989). Molecular evidence of genetic heterogeneity in Wistar-Kyoto rats: implications for research with the spontaneously hypertensive rat. Hypertens. *13*: 188–192.

Kuzmin, A., Sandin, J., Terenius, L., and Ogren, S.O. (2000). Dose- and time-dependent bimodal effects of kappa-opioid agonists on locomotor activity in mice. J. Pharmacol. Exp. Ther. *295*: 1031–1042.

Kwon, M., Altin, M., Duenas, H., and Alev, L. (2014). The role of descending inhibitory pathways on chronic pain modulation and clinical implications. Pain Pract. *14*: 656–667.

La Garza, R. De, and Mahoney, J.J. (2004). A distinct neurochemical profile in WKY rats at baseline and in response to acute stress: implications for animal models of anxiety and depression. Brain Res. *1021*: 209–18.

Labuda, C.J., and Fuchs, P.N. (2000). A behavioral test paradigm to measure the aversive quality of inflammatory and neuropathic pain in rats. Exp. Neurol. *163*: 490–494.

Labuda, C.J., and Fuchs, P.N. (2001). Low dose aspirin attenuates escape/avoidance behavior, but does not reduce mechanical hyperalgesia in a rodent model of inflammatory pain. Neurosci. Lett. *304*: 137–140.

LaBuda, C.J., and Fuchs, P.N. (2005). Attenuation of negative pain affect produced by unilateral spinal nerve injury in the rat following anterior cingulate cortex activation. Neuroscience *136*: 311–322.

Labus, J.S., Dinov, I.D., Jiang, Z., Ashe-McNalley, C., Zamanyan, A., Shi, Y., et al. (2014). Irritable bowel syndrome in female patients is associated with alterations in structural brain networks. Pain *155*: 137–149.

LaGraize, S.C., Labuda, C.J., Rutledge, M.A., Jackson, R.L., and Fuchs, P.N. (2004). Differential effect of anterior cingulate cortex lesion on mechanical hypersensitivity and escape/avoidance behavior in an animal model of neuropathic pain. Exp. Neurol. *188*: 139–148.

Lahat, A., Hong, M., and Fox, N.A. (2011). Behavioural inhibition: is it a risk factor for anxiety? Int. Rev. Psychiatry *23*: 248–257.

Lahmame, A., Arco, C. Del, Pazos, A., Yritia, M., and Armario, A. (1997). Are Wistar-Kyoto rats a genetic animal model of depression resistant to antidepressants? Eur. J. Pharmacol. *337*: 115–123.

Lahti, R.A., VonVoigtlander, P.F., and Barsuhn, C. (1982). Properties of a selective kappa agonist, U-50,488H. Life Sci. *31*: 2257–2260.

Laird, J.M., and Cervero, F. (1990). Tonic descending influences on receptive-field properties of nociceptive dorsal horn neurons in sacral spinal cord of rat. J. Neurophysiol. *63*: 1022–1032.

Land, B.B., Bruchas, M.R., Lemos, J.C., Xu, M., Melief, E.J., and Chavkin, C. (2008). The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. J. Neurosci. *28*: 407–14.

Land, B.B., Bruchas, M.R., Schattauer, S., Giardino, W.J., Aita, M., Messinger, D., et al. (2009). Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. Proc. Natl. Acad. Sci. U. S. A. *106*: 19168–19173.

Lane, D.A., Patel, P.A., and Morgan, M.M. (2005). Evidence for an intrinsic mechanism of antinociceptive tolerance within the ventrolateral periaqueductal gray of rats. Neuroscience *135*: 227–234.

Langen, B., and Dost, R. (2011). Comparison of SHR, WKY and Wistar rats in different behavioural animal models: Effect of dopamine D1 and alpha2 agonists. ADHD Atten. Deficit Hyperact. Disord. *3*: 1–12.

Lau, B.K., and Vaughan, C.W. (2014). Descending modulation of pain: The GABA disinhibition hypothesis of analgesia. Curr. Opin. Neurobiol. *29*: 159–164.

Lautenbacher, S., Spernal, J., Schreiber, W., and Krieg, J.C. (1999). Relationship between clinical pain complaints and pain sensitivity in patients with depression and panic disorder. Psychosom. Med. *61*: 822–827.

Law, P.Y., Wong, Y.H., and Loh, H.H. (2000). Molecular mechanisms and regulation of opioid receptor signaling. Annu. Rev. Pharmacol. Toxicol. *40*: 389–430.

Leah, J.D., Sandkuhler, J., Herdegen, T., Murashov, A., and Zimmermann, M. (1992). Potentiated expression of FOS protein in the rat spinal cord following bilateral noxious cutaneous stimulation. Neuroscience *48*: 525–532.

Lee, U.J., Ackerman, A.L., Wu, A., Zhang, R., Leung, J., Bradesi, S., et al. (2015). Chronic psychological stress in high-anxiety rats induces sustained bladder hyperalgesia. Physiol. Behav. *139*: 541–548.

Lei, L.-G., Zhang, Y.-Q., and Zhao, Z.-Q. (2004). Pain-related aversion and Fos expression in the central nervous system in rats. Neuroreport *15*: 67–71.

Leite-Almeida, H., Almeida-Torres, L., Mesquita, A.R., Pertovaara, A., Sousa, N., Cerqueira, J.J., et al. (2009). The impact of age on emotional and cognitive behaviours triggered by experimental neuropathy in rats. Pain *144*: 57–65.

Leite-Almeida, H., Cerqueira, J.J., Wei, H., Ribeiro-Costa, N., Anjos-Martins, H., Sousa, N., et al. (2012). Differential effects of left/right neuropathy on rats' anxiety and cognitive behavior. Pain *153*: 2218–2225.

Lemos, J.C., Roth, C.A., Messinger, D.I., Gill, H.K., Phillips, P.E.M., and Chavkin, C. (2012). Repeated stress dysregulates kappa-opioid receptor signaling in the dorsal raphe through a p38alpha MAPK-dependent mechanism. J. Neurosci. *32*: 12325–12336.

Lever, J.R., Scheffel, U., Kinter, C.M., Ravert, H.T., Dannals, R.F., Wagner, H.N., et al. (1992). In vivo binding of N1′-([11C]methyl)naltrindole to δ-opioid receptors in mouse brain. Eur. J. Pharmacol. *216*: 459–460.

Lewis, V.A., and Gebhart, G.F. (1977). Morphine-induced and stimulation-produced analgesias at coincident periaqueductal central gray loci: evaluation of analgesic congruence, tolerance, and cross-tolerance. Exp. Neurol. *57*: 934–955.

Li, C.H., and Chung, D. (1976). Isolation and structure of an untriakontapeptide with opiate activity from camel pituitary glands. Proc. Natl. Acad. Sci. U. S. A. *73*: 1145–1148.

Li, J.-X. (2015). Pain and depression comorbidity: a preclinical perspective. Behav. Brain

Res. *276*: 92–8.

Li, M.-J., Liu, L.-Y., Chen, L., Cai, J., Wan, Y., and Xing, G.-G. (2017). Chronic stress exacerbates neuropathic pain via the integration of stress-affect-related information with nociceptive information in the central nucleus of the amygdala. Pain *158*: 717–739.

Li, Q., Yue, N., Liu, S.-B., Wang, Z.-F., Mi, W.-L., Jiang, J.-W., et al. (2014). Effects of chronic electroacupuncture on depression- and anxiety-like behaviors in rats with chronic neuropathic pain. Evid. Based. Complement. Alternat. Med. *2014*: 158987.

Li, Y., Peng, S., Wan, C., Cao, L., and Li, Y. (2005). [Chronic pain impairs spatial learning and memory ability and down-regulates Bcl-2 and BDNF mRNA expression in hippocampus of neonatal rats]. Zhonghua er ke za zhi = Chinese J. Pediatr. *43*: 444–8.

Liang, D.-Y., Li, X., and Clark, J.D. (2011). 5-hydroxytryptamine type 3 receptor modulates opioid-induced hyperalgesia and tolerance in mice. Anesthesiology *114*: 1180–1189.

Lima, D., Mendes-Ribeiro, J.A., and Coimbra, A. (1991). The spino-latero-reticular system of the rat: Projections from the superficial dorsal horn and structural characterization of marginal neurons involved. Neuroscience *45*: 137–152.

Lindner, M.D., Plone, M.A., Francis, J.M., and Cain, C.K. (1999). Chronic morphine reduces pain-related disability in a rodent model of chronic, inflammatory pain. Exp. Clin. Psychopharmacol. *7*: 187–197.

Linton, S.J. (2000). A review of psychological risk factors in back and neck pain. Spine (Phila. Pa. 1976). *25*: 1148–1156.

Liu, H.C., Lu, S., Augustin, L.B., Felsheim, R.F., Chen, H.C., Loh, H.H., et al. (1995). Cloning and promoter mapping of mouse kappa opioid receptor gene. Biochem. Biophys. Res. Commun. *209*: 639–647.

Liu, L.Y., Zhang, R.L., Chen, L., Zhao, H.Y., Cai, J., Wang, J.K., et al. (2019a). Chronic stress increases pain sensitivity via activation of the rACC–BLA pathway in rats. Exp. Neurol. *313*: 109–123.

Liu, M.G., and Chen, J. (2014). Preclinical research on pain comorbidity with affective disorders and cognitive deficits: Challenges and perspectives. Prog. Neurobiol. *116*: 13–32.

Liu, S.S., Pickens, S., Burma, N.E., Ibarra-Lecue, I., Yang, H., Xue, L., et al. (2019b). Kappa Opioid Receptors Drive a Tonic Aversive Component of Chronic Pain. J. Neurosci. *39*: 4162–4178.

Liu, W., Ge, T., Leng, Y., Pan, Z., Fan, J., Yang, W., et al. (2017). The Role of Neural Plasticity in Depression: From Hippocampus to Prefrontal Cortex. Neural Plast. *2017*: 6871089.

Liu, Y., Yang, L., Yu, J., and Zhang, Y.Q. (2015). Persistent, comorbid pain and anxiety can be uncoupled in a mouse model. Physiol. Behav. *151*: 55–63.

Llewelyn, M.B., Azami, J., Gibbs, M., and Roberts, M.H. (1983). A comparison of the sites at which pentazocine and morphine act to produce analgesia. Pain *16*: 313–331.

Lohith, T.G., Zoghbi, S.S., Morse, C.L., Araneta, M.F., Barth, V.N., Goebl, N.A., et al. (2012). Brain and whole-body imaging of nociceptin/orphanin FQ peptide receptor in humans using the PET ligand 11C-NOP-1A. J. Nucl. Med. *53*: 385–392.

Lopez-Rubalcava, C., and Lucki, I. (2000). Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test. Neuropsychopharmacology *22*: 191–199.

Lord, J.A., Waterfield, A.A., Hughes, J., and Kosterlitz, H.W. (1977). Endogenous opioid peptides: multiple agonists and receptors. Nature *267*: 495–499.

Low, L.A., Millecamps, M., Seminowicz, D.A., Naso, L., Thompson, S.J., Stone, L.S., et al. (2012). Nerve injury causes long-term attentional deficits in rats. Neurosci. Lett. *529*: 103–107.

Loyd, D.R., Wang, X., and Murphy, A.Z. (2008). Sex differences in micro-opioid receptor expression in the rat midbrain periaqueductal gray are essential for eliciting sex differences in morphine analgesia. J. Neurosci. *28*: 14007–14017.

Lu, Y., Zhu, L., and Gao, Y.J. (2011). Pain-related aversion induces astrocytic reaction and proinflammatory cytokine expression in the anterior cingulate cortex in rats. Brain Res. Bull. *84*: 178–182.

Lueptow, L.M., Fakira, A.K., and Bobeck, E.N. (2018). The Contribution of the Descending Pain Modulatory Pathway in Opioid Tolerance. Front. Neurosci. *12*: 886.

Lutz, P.E., and Kieffer, B.L. (2013). Opioid receptors: Distinct roles in mood disorders. Trends Neurosci. *36*: 195–206.

Ma, Q.P., and Han, J.S. (1991). Naloxone blocks the release of opioid peptides in periaqueductal gray and N. accumbens induced by intra-amygdaloid injection of morphine. Peptides *12*: 1235–1238.

Ma, W., and Peschanski, M. (1988). Spinal and trigeminal projections to the parabrachial nucleus in the rat: electron-microscopic evidence of a spino-ponto-amygdalian somatosensory pathway. Somatosens. Res. *5*: 247–257.

Maciel, I.S., Silva, R.B.M., Morrone, F.B., Calixto, J.B., and Campos, M.M. (2013). Synergistic effects of celecoxib and bupropion in a model of chronic inflammation-related depression in mice. PLoS One *8*: e77227.

MacLean, K.A., Johnson, M.W., Reissig, C.J., Prisinzano, T.E., and Griffiths, R.R. (2013). Dose-related effects of salvinorin A in humans: dissociative, hallucinogenic, and memory effects. Psychopharmacology (Berl). *226*: 381–392.

Madasu, M.K., Okine, B.N., Olango, W.M., Rea, K., Lenihan, R., Roche, M., et al. (2016). Genotype-dependent responsivity to inflammatory pain: A role for TRPV1 in the periaqueductal grey. Pharmacol. Res. *113*: 44–54.

Mague, S.D., Pliakas, A.M., Todtenkopf, M.S., Tomasiewicz, H.C., Zhang, Y., Stevens, W.C.J., et al. (2003). Antidepressant-like effects of kappa-opioid receptor antagonists in the forced swim test in rats. J. Pharmacol. Exp. Ther. *305*: 323–330.

Mahinda, T.B., Lovell, B.M., and Taylor, B.K. (2004). Morphine-induced analgesia, hypotension, and bradycardia are enhanced in hypertensive rats. Anesth. Analg. *98*: 1698–704, table of contents.

Malan, T.P., Ossipov, M.H., Gardell, L.R., Ibrahim, M., Bian, D., Lai, J., et al. (2000). Extraterritorial neuropathic pain correlates with multisegmental elevation of spinal dynorphin in nerve-injured rats. Pain *86*: 185–194.

Maleki, N., Becerra, L., Brawn, J., McEwen, B., Burstein, R., and Borsook, D. (2013). Common hippocampal structural and functional changes in migraine. Brain Struct. Funct.

218: 903–912.

Malkesman, O., Braw, Y., Zagoory-Sharon, O., Golan, O., Lavi-Avnon, Y., Schroeder, M., et al. (2005). Reward and anxiety in genetic animal models of childhood depression. Behav. Brain Res. *164*: 1–10.

Malkesman, O., Maayan, R., Weizman, A., and Weller, A. (2006). Aggressive behavior and HPA axis hormones after social isolation in adult rats of two different genetic animal models for depression. Behav. Brain Res. *175*: 408–414.

Malkesman, O., and Weller, A. (2009). Two different putative genetic animal models of childhood depression-A review. Prog. Neurobiol. *88*: 153–169.

Malmberg, A.B., and Yaksh, T.L. (1994). Capsaicin-evoked prostaglandin E2 release in spinal cord slices: relative effect of cyclooxygenase inhibitors. Eur. J. Pharmacol. *271*: 293–299.

Manabe, T., Noda, Y., Mamiya, T., Katagiri, H., Houtani, T., Nishi, M., et al. (1998). Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors. Nature *394*: 577–581.

Manglik, A., Kruse, A.C., Kobilka, T.S., Thian, F.S., Mathiesen, J.M., Sunahara, R.K., et al. (2012). Crystal structure of the mu-opioid receptor bound to a morphinan antagonist. Nature *485*: 321–326.

Manning, B.H., and Mayer, D.J. (1995a). The central nucleus of the amygdala contributes to the production of morphine antinociception in the formalin test. Pain *63*: 141–52.

Manning, B.H., and Mayer, D.J. (1995b). The central nucleus of the amygdala contributes to the production of morphine antinociception in the rat tail-flick test. J. Neurosci. *15*: 8199–8213.

Manning, B.H., Morgan, M.J., and Franklin, K.B. (1994). Morphine analgesia in the formalin test: evidence for forebrain and midbrain sites of action. Neuroscience *63*: 289–94.

Mansour, A., Fox, C.A., Akil, H., and Watson, S.J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci. *18*: 22–29.

Mansour, A., Khachaturian, H., Lewis, M., Akil, H., and Watson, S. (1987). Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. J. Neurosci. *7*: 2445–2464.

Mansour, A., Lewis, M.E., Khachaturian, H., Akil, H., and Watson, S.J. (1986). Pharmacological and anatomical evidence of selective μ, δ, and χ opioid receptor binding in rat brain. Brain Res. *399*: 69–79.

Mantyh, P.W. (1983). Connections of midbrain periaqueductal gray in the monkey. II. Descending efferent projections. J. Neurophysiol. *49*: 582–594.

Mao, C.P., and Yang, H.J. (2015). Smaller Amygdala Volumes in Patients with Chronic Low Back Pain Compared with Healthy Control Individuals. J. Pain *16*: 1366–1376.

Margolis, E.B., and Karkhanis, A.N. (2019). Dopaminergic cellular and circuit contributions to kappa opioid receptor mediated aversion. Neurochem. Int. *129*: 104504.

Márki, Á., Monory, K., Ötvös, F., Tóth, G., Krassnig, R., Schmidhammer, H., et al. (1999). μ-Opioid receptor specific antagonist cyprodime: Characterization by in vitro

radioligand and [35S]GTPγS binding assays. Eur. J. Pharmacol. *383*: 209–214.

Martenson, M.E., Cetas, J.S., and Heinricher, M.M. (2009). A possible neural basis for stress-induced hyperalgesia. Pain *142*: 236–244.

Martikainen, I.K., Pecina, M., Love, T.M., Nuechterlein, E.B., Cummiford, C.M., Green, C.R., et al. (2013). Alterations in Endogenous Opioid Functional Measures in Chronic Back Pain. J. Neurosci. *33*: 14729–14737.

Martín del Campo, A.F., McMurray, R.G., Besser, G.M., and Grossman, A. (1992). Effect of 12-hour infusion of naloxone on mood and cognition in normal male volunteers. Biol. Psychiatry *32*: 344–353.

Martin, M., Matifas, A., Maldonado, R., and Kieffer, B.L. (2003). Acute antinociceptive responses in single and combinatorial opioid receptor knockout mice: distinct mu, delta and kappa tones. Eur. J. Neurosci. *17*: 701–708.

Marvizon, J.C.G., Chen, W., and Murphy, N. (2009). Enkephalins, dynorphins, and beta-endorphin in the rat dorsal horn: an immunofluorescence colocalization study. J. Comp. Neurol. *517*: 51–68.

Massaly, N., Copits, B.A., Wilson-Poe, A.R., Hipólito, L., Markovic, T., Yoon, H.J., et al. (2019). Pain-Induced Negative Affect Is Mediated via Recruitment of The Nucleus Accumbens Kappa Opioid System. Neuron *102*: 564-573.e6.

Matsuzawa-Yanagida, K., Narita, M., Nakajima, M., Kuzumaki, N., Niikura, K., Nozaki, H., et al. (2008). Usefulness of antidepressants for improving the neuropathic pain-like state and pain-induced anxiety through actions at different brain sites. Neuropsychopharmacology *33*: 1952–1965.

Matthes, H.W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature *383*: 819–823.

Matthies, B.K., and Franklin, K.B. (1992). Formalin pain is expressed in decerebrate rats but not attenuated by morphine. Pain *51*: 199–206.

Matthies, H., Schroeder, H., Becker, A., Loh, H., Höllt, V., and Krug, M. (2000). Lack of expression of long-term potentiation in the dentate gyrus but not in the CA1 region of the hippocampus of μ-opioid receptor-deficient mice. Neuropharmacology *39*: 952–960.

Matus-Ortega, M.E., Leff Gelman, P., Calva-Nieves, J.C., Flores-Zamora, A., Salazar-Juarez, A., Torner-Aguilar, C.-A., et al. (2017). Mexneurin is a novel precursor of peptides in the central nervous system of rodents. FEBS Lett. *591*: 1627–1636.

Max, M.B., Wu, T., Atlas, S.J., Edwards, R.R., Haythornthwaite, J.A., Bollettino, A.F., et al. (2006). A clinical genetic method to identify mechanisms by which pain causes depression and anxiety. Mol. Pain *2*: 1–15.

Mayer, D.J., Wolfle, T.L., Akil, H., Carder, B., and Liebeskind, J.C. (1971). Analgesia from electrical stimulation in the brainstem of the rat. Science *174*: 1351–1354.

McAuley, J.D., Stewart, A.L., Webber, E.S., Cromwell, H.C., Servatius, R.J., and Pang, K.C.H. (2009). Wistar-Kyoto rats as an animal model of anxiety vulnerability: Support for a hypervigilance hypothesis. Behav. Brain Res. *204*: 162–168.

McCormack, K., Prather, P., and Chapleo, C. (1998). Some new insights into the effects of opioids in phasic and tonic nociceptive tests. Pain *78*: 79–98.

McDonald, A.J. (1987). Organization of amygdaloid projections to the mediodorsal thalamus and prefrontal cortex: a fluorescence retrograde transport study in the rat. J. Comp. Neurol. *262*: 46–58.

McEwen, B.S. (2007). Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol. Rev. *87*: 873–904.

McGaraughty, S., Chu, K.L., Bitner, R.S., Martino, B., Kouhen, R. El, Han, P., et al. (2003). Capsaicin infused into the PAG affects rat tail flick responses to noxious heat and alters neuronal firing in the RVM. J. Neurophysiol. *90*: 2702–2710.

McGaraughty, S., Reinis, S., and Tsoukatos, J. (1993). Two distinct unit activity responses to morphine in the rostral ventromedial medulla of awake rats. Brain Res. *604*: 331–333.

McGivern, R.F., Mousa, S., Couri, D., and Berntson, G.G. (1983). Prolonged intermittent footshock stress decreases Met and Leu enkephalin levels in brain with concomitant decreases in pain threshold. Life Sci. *33*: 47–54.

McHugh, K. (2019). Exploration of the opioidergic system in the olfactory bulbectomized rat model of depression. PhD thesis. National University of Ireland Galway.

McIlwain, K.L., Merriweather, M.Y., Yuva-Paylor, L.A., and Paylor, R. (2001). The use of behavioral test batteries: Effects of training history. Physiol. Behav. *73*: 705–717.

McLaughlin, J.P., Marton-Popovici, M., and Chavkin, C. (2003). Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J. Neurosci. *23*: 5674–83.

McNamara, C.R., Mandel-Brehm, J., Bautista, D.M., Siemens, J., Deranian, K.L., Zhao, M., et al. (2007). TRPA1 mediates formalin-induced pain. Proc. Natl. Acad. Sci. U. S. A. *104*: 13525–13530.

McWilliams, L.A., Cox, B.J., and Enns, M.W. (2003). Mood and anxiety disorders associated with chronic pain: an examination in a nationally representative sample. Pain *106*: 127–133.

McWilliams, L.A., Goodwin, R.D., and Cox, B.J. (2004). Depression and anxiety associated with three pain conditions: results from a nationally representative sample. Pain *111*: 77–83.

Mehalick, M.L., Ingram, S.L., Aicher, S.A., and Morgan, M.M. (2013). Chronic inflammatory pain prevents tolerance to the antinociceptive effect of morphine microinjected into the ventrolateral periaqueductal gray of the rat. J. Pain *14*: 1601–1610.

Meilandt, W.J., Barea-Rodriguez, E., Harvey, S.A.K., and Martinez, J.L.J. (2004). Role of hippocampal CA3 mu-opioid receptors in spatial learning and memory. J. Neurosci. *24*: 2953–2962.

Meints, S.M., and Edwards, R.R. (2018). Evaluating psychosocial contributions to chronic pain outcomes. Prog. Neuro-Psychopharmacology Biol. Psychiatry *87*: 168–182.

Melichar, J.K., Malizia, A.L., and Nutt, D.J. (1997). Organization of Opioid Receptors in Human Brain and Drug Interactions Studied by PET and SPECT Imaging: Implications for Treatment Strategies for Opiate Addiction. Semin. Neurosci. *9*: 131–139.

Melief, E.J., Miyatake, M., Bruchas, M.R., and Chavkin, C. (2010). Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. Proc. Natl. Acad. Sci. U. S. A. *107*: 11608–11613.

Melzack, R., and Casey, K. (1968). Sensory, motivational, and central control determinants of pain: a new conceptual model in pain. In The Skin Senses: Proceedings., D.R. Kenshalo, ed. (Springfield, IL: Charles C Thomas), pp 423–439.

Melzack, R., and Wall, P.D. (1965). Pain mechanisms: a new theory. Science *150*: 971–979.

Ménard, C., Tse, Y.C., Cavanagh, C., Chabot, J.-G., Herzog, H., Schwarzer, C., et al. (2013). Knockdown of prodynorphin gene prevents cognitive decline, reduces anxiety, and rescues loss of group 1 metabotropic glutamate receptor function in aging. J. Neurosci. *33*: 12792–12804.

Mendes-Gomes, J., Amaral, V.C.S., and Nunes-de-Souza, R.L. (2011). Ventrolateral periaqueductal gray lesion attenuates nociception but does not change anxiety-like indices or fear-induced antinociception in mice. Behav. Brain Res. *219*: 248–253.

Menétrey, D., Gannon, A., Levine, J.D., and Basbaum, A.I. (1989). Expression of c-fos protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular, and visceral stimulation. J. Comp. Neurol. *285*: 177–195.

Meng, F., Xie, G.X., Thompson, R.C., Mansour, A., Goldstein, A., Watson, S.J., et al. (1993). Cloning and pharmacological characterization of a rat kappa opioid receptor. Proc. Natl. Acad. Sci. U. S. A. *90*: 9954–9958.

Meng, I.D., and Harasawa, I. (2007). Chronic morphine exposure increases the proportion of on-cells in the rostral ventromedial medulla in rats. Life Sci. *80*: 1915–1920.

Merrer, J. Le, Becker, J.A.J., Befort, K., and Kieffer, B.L. (2009). Reward processing by the opioid system in the brain. Physiol. Rev. *89*: 1379–1412.

Merrer, J. Le, Plaza-Zabala, A., Boca, C. Del, Matifas, A., Maldonado, R., and Kieffer, B.L. (2011). Deletion of the δ opioid receptor gene impairs place conditioning but preserves morphine reinforcement. Biol. Psychiatry *69*: 700–703.

Merrer, J. Le, Rezai, X., Scherrer, G., Becker, J.A.J., and Kieffer, B.L. (2013). Impaired hippocampus-dependent and facilitated striatum-dependent behaviors in mice lacking the δ opioid receptor. Neuropsychopharmacology *38*: 1050–9.

Metz, A.E., Yau, H.-J., Centeno, M.V., Apkarian, A.V., and Martina, M. (2009). Morphological and functional reorganization of rat medial prefrontal cortex in neuropathic pain. Proc. Natl. Acad. Sci. U. S. A. *106*: 2423–2428.

Meunier, J.C. (1997). Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. Eur. J. Pharmacol. *340*: 1–15.

Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., et al. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature *377*: 532–535.

Michelotti, A., Farella, M., Tedesco, A., Cimino, R., and Martina, R. (2000). Changes in pressure-pain thresholds of the jaw muscles during a natural stressful condition in a group of symptom-free subjects. J. Orofac. Pain *14*: 279–285.

Mignat, C., Wille, U., and Ziegler, A. (1995). Affinity profiles of morphine, codeine, dihydrocodeine and their glucuronides at opioid receptor subtypes. Life Sci. *56*: 793–799.

Mika, J., Przewlocki, R., and Przewlocka, B. (2001). The role of δ-opioid receptor subtypes in neuropathic pain. Eur. J. Pharmacol. *415*: 31–37.

Millan, M.J. (1999). The induction of pain: an integrative review. Prog. Neurobiol. *57*: 1–164.

Millan, M.J. (2002). Descending control of pain. Prog. Neurobiol. *66*: 355–474.

Millan, M.J., Czlonkowski, A., Pilcher, C.W., Almeida, O.F., Millan, M.H., Colpaert, F.C., et al. (1987). A model of chronic pain in the rat: functional correlates of alterations in the activity of opioid systems. J. Neurosci. *7*: 77–87.

Min, B.H., Augustin, L.B., Felsheim, R.F., Fuchs, J.A., and Loh, H.H. (1994). Genomic structure analysis of promoter sequence of a mouse mu opioid receptor gene. Proc. Natl. Acad. Sci. U. S. A. *91*: 9081–9085.

Minami, M., Hosoi, Y., Toya, T., Katao, Y., Maekawa, K., Katsumata, S., et al. (1993). In situ hybridization study of κ-opioid receptor mRNA in the rat brain. Neurosci. Lett. *162*: 161–164.

Miranda-Páez, A., Zamudio, S., Vázquez-León, P., Campos-Rodríguez, C., and Ramírez-San Juan, E. (2016). Involvement of opioid and GABA systems in the ventrolateral periaqueductal gray on analgesia associated with tonic immobility. Pharmacol. Biochem. Behav. *142*: 72–78.

Mizoguchi, H., Fukumoto, K., Sakamoto, G., Jin, S., Toyama, A., Wang, T., et al. (2019). Maternal separation as a risk factor for aggravation of neuropathic pain in later life in mice. Behav. Brain Res. *359*: 942–949.

Mogil, J.S. (2012). Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. Nat. Rev. Neurosci. *13*: 859–866.

Mogil, J.S., Chesler, E.J., Wilson, S.G., Juraska, J.M., and Sternberg, W.F. (2000). Sex differences in thermal nociception and morphine antinociception in rodents depend on genotype. Neurosci. Biobehav. Rev. *24*: 375–89.

Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., et al. (1994). ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett. *341*: 33–38.

Monassi, C.R., Ramos Andrade Leite-Panissi, C., and Menescal-de-Oliveira, L. (1999). Ventrolateral periaqueductal gray matter and the control of tonic immobility. Brain Res. Bull. *50*: 201–208.

Moon, S.W., Park, E.H., Suh, H.R., Ko, D.H., Kim, Y.I., and Han, H.C. (2016). The contribution of activated peripheral kappa opioid receptors (kORs) in the inflamed knee joint to anti-nociception. Brain Res. *1648*: 11–18.

Moore, D.S., McCabe, G.P., and Craig, B.A. (2009). Introduction to the Practice of Statistics (New York: W.H. Freeman and Company).

Moreau, J.L., and Fields, H.L. (1986). Evidence for GABA involvement in midbrain control of medullary neurons that modulate nociceptive transmission. Brain Res. *397*: 37–46.

Morgan, M.M., Bobeck, E.N., and Ingram, S.L. (2009). Glutamate modulation of antinociception, but not tolerance, produced by morphine microinjection into the periaqueductal gray of the rat. Brain Res. *1295*: 59–66.

Morgan, M.M., and Clayton, C.C. (2005). Defensive behaviors evoked from the ventrolateral periaqueductal gray of the rat: Comparison of opioid and GABA disinhibition. Behav. Brain Res. *164*: 61–66.

Morgan, M.M., Clayton, C.C., and Lane, D.A. (2003). Behavioral evidence linking opioid-sensitive GABAergic neurons in the ventrolateral periaqueductal gray to morphine tolerance. Neuroscience *118*: 227–232.

Morgan, M.M., Fossum, E.N., Stalding, B.M., and King, M.M. (2006). Morphine Antinociceptive Potency on Chemical, Mechanical, and Thermal Nociceptive Tests in the Rat. J. Pain *7*: 358–366.

Morgan, M.M., Whitney, P.K., and Gold, M.S. (1998). Immobility and flight associated with antinociception produced by activation of the ventral and lateral/dorsal regions of the rat periaqueductal gray. Brain Res. *804*: 159–166.

Moriarty, O., and Finn, D.P. (2014). Cognition and pain. Curr. Opin. Support. Palliat. Care *8*: 130–136.

Moriarty, O., McGuire, B.E., and Finn, D.P. (2011). The effect of pain on cognitive function: A review of clinical and preclinical research. Prog. Neurobiol. *93*: 385–404.

Moriarty, O., Roche, M., McGuire, B.E., and Finn, D.P. (2012). Validation of an air-puff passive-avoidance paradigm for assessment of aversive learning and memory in rat models of chronic pain. J. Neurosci. Methods *204*: 1–8.

Moskowitz, A.S., and Goodman, R.R. (1984). Light microscopic autoradiographic localization of mu and delta opioid binding sites in the mouse central nervous system. J. Neurosci. *4*: 1331–1342.

Motta, V., and Brandao, M.L. (1993). Aversive and antiaversive effects of morphine in the dorsal periaqueductal gray of rats submitted to the elevated plus-maze test. Pharmacol. Biochem. Behav. *44*: 119–125.

Moye, L.S., Tipton, A.F., Dripps, I., Sheets, Z., Crombie, A., Violin, J.D., et al. (2019). Delta opioid receptor agonists are effective for multiple types of headache disorders. Neuropharmacology *148*: 77–86.

Mudge, A.W., Leeman, S.E., and Fischbach, G.D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. Proc. Natl. Acad. Sci. U. S. A. *76*: 526–530.

Mukae, T., Uchida, H., and Ueda, H. (2015). Donepezil Reverses Intermittent Stress-Induced Generalized Chronic Pain Syndrome in Mice. J. Pharmacol. Exp. Ther. *353*: 471–479.

Munro, G. (2009). Pharmacological assessment of the rat formalin test utilizing the clinically used analgesic drugs gabapentin, lamotrigine, morphine, duloxetine, tramadol and ibuprofen: Influence of low and high formalin concentrations. Eur. J. Pharmacol. *605*: 95–102.

Munro, G., Dyhr, H., and Grunnet, M. (2010). Selective potentiation of gabapentin-mediated antinociception in the rat formalin test by the nicotinic acetylcholine receptor agonist ABT-594. Neuropharmacology *59*: 208–217.

Munro, G., Storm, A., Hansen, M.K., Dyhr, H., Marcher, L., Erichsen, H.K., et al. (2012). The combined predictive capacity of rat models of algogen-induced and neuropathic hypersensitivity to clinically used analgesics varies with nociceptive endpoint and consideration of locomotor function. Pharmacol. Biochem. Behav. *101*: 465–478.

Murthy, K.S., and Makhlouf, G.M. (1996). Opioid mu, delta, and kappa receptor-induced activation of phospholipase C-beta 3 and inhibition of adenylyl cyclase is mediated by

Gi2 and G(o) in smooth muscle. Mol. Pharmacol. *50*: 870–877.

Mutso, A.A., Radzicki, D., Baliki, M.N., Huang, L., Banisadr, G., Centeno, M. V, et al. (2012). Abnormalities in hippocampal functioning with persistent pain. J. Neurosci. *32*: 5747–5756.

Nadal, X., Banos, J.-E., Kieffer, B.L., and Maldonado, R. (2006). Neuropathic pain is enhanced in delta-opioid receptor knockout mice. Eur. J. Neurosci. *23*: 830–834.

Naidu, P.S., Lichtman, A.H., Archer, C.C., May, E.L., Harris, L.S., and Aceto, M.D. (2007). NIH 11082 produces anti-depressant-like activity in the mouse tail-suspension test through a delta-opioid receptor mechanism of action. Eur. J. Pharmacol. *566*: 132–136.

Nam, H., Clinton, S.M., Jackson, N.L., and Kerman, I.A. (2014). Learned helplessness and social avoidance in the Wistar-Kyoto rat. Front. Behav. Neurosci. *8*: 1–18.

Nappi, G., Facchinetti, F., Martignoni, E., Petraglia, F., Bono, G., and Genazzani, A.R. (1985). CSF beta-EP in headache and depression. Cephalalgia *5*: 99–101.

Narita, M., Kaneko, C., Miyoshi, K., Nagumo, Y., Kuzumaki, N., Nakajima, M., et al. (2006a). Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. Neuropsychopharmacology *31*: 739–50.

Narita, M., Kuzumaki, N., Narita, M., Kaneko, C., Hareyama, N., Miyatake, M., et al. (2006b). Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical δ-opioid receptor dysfunction. J. Neurochem. *97*: 1369–1378.

Nascimento, G.C. do, and Leite-Panissi, C.R.A. (2014). Time-dependent analysis of nociception and anxiety-like behavior in rats submitted to persistent inflammation of the temporomandibular joint. Physiol. Behav. *125*: 1–7.

Nathan, P.W., Smith, M., and Deacon, P. (2001). The crossing of the spinothalamic tract. Brain *124*: 793–803.

Navratilova, E., Ji, G., Phelps, C., Qu, C., Hein, M., Yakhnitsa, V., et al. (2019). Kappa opioid signaling in the central nucleus of the amygdala promotes disinhibition and aversiveness of chronic neuropathic pain. Pain *160*: 824–832.

Neal, C.R.J., Mansour, A., Reinscheid, R., Nothacker, H.P., Civelli, O., Akil, H., et al. (1999). Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding. J. Comp. Neurol. *412*: 563–605.

Negrete, R., García Gutiérrez, M.S., Manzanares, J., and Maldonado, R. (2017). Involvement of the dynorphin/KOR system on the nociceptive, emotional and cognitive manifestations of joint pain in mice. Neuropharmacology *116*: 315–327.

Neubert, M.J., Kincaid, W., and Heinricher, M.M. (2004). Nociceptive facilitating neurons in the rostral ventromedial medulla. Pain *110*: 158–165.

Neugebauer, V., Li, W., Bird, G.C., and Han, J.S. (2004). The amygdala and persistent pain. Neuroscientist *10*: 221–34.

Newton, S.S., Thome, J., Wallace, T.L., Shirayama, Y., Schlesinger, L., Sakai, N., et al. (2002). Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. J. Neurosci. *22*: 10883–10890.

Niddam, D.M., Chan, R.C., Lee, S.H., Yeh, T.C., and Hsieh, J.C. (2008). Central

representation of hyperalgesia from myofascial trigger point. Neuroimage *39*: 1299–1306.

Niddam, D.M., Lee, S.-H., Su, Y.-T., and Chan, R.-C. (2017). Brain structural changes in patients with chronic myofascial pain. Eur. J. Pain *21*: 148–158.

Nilsen, K.B., Sand, T., Westgaard, R.H., Stovner, L.J., White, L.R., Bang Leistad, R., et al. (2007). Autonomic activation and pain in response to low-grade mental stress in fibromyalgia and shoulder/neck pain patients. Eur. J. Pain *11*: 743–755.

Nishinaka, T., Nakamoto, K., and Tokuyama, S. (2016). Early life stress induces sex-dependent increases in phosphorylated extracellular signal-regulated kinase in brains of mice with neuropathic pain. Eur. J. Pain *20*: 1346–1356.

Nishiyori, M., Nagai, J., Nakazawa, T., and Ueda, H. (2010). Absence of morphine analgesia and its underlying descending serotonergic activation in an experimental mouse model of fibromyalgia. Neurosci. Lett. *472*: 184–187.

Noble, F., Banisadr, G., Jardinaud, F., Popovici, T., Lai-Kuen, R., Chen, H., et al. (2001). First discrete autoradiographic distribution of aminopeptidase N in various structures of rat brain and spinal cord using the selective iodinated inhibitor [125I]RB 129. Neuroscience *105*: 479–488.

Norman, G.J., Karelina, K., Zhang, N., Walton, J.C., Morris, J.S., and Devries, A.C. (2010). Stress and IL-1beta contribute to the development of depressive-like behavior following peripheral nerve injury. Mol. Psychiatry *15*: 404–414.

North, R.A., and Williams, J.T. (1985). On the potassium conductance increased by opioids in rat locus coeruleus neurones. J. Physiol. *364*: 265–280.

Nosek, K., Dennis, K., Andrus, B.M., Ahmadiyeh, N., Baum, A.E., Solberg Woods, L.C., et al. (2008). Context and strain-dependent behavioral response to stress. Behav. Brain Funct. *4*: 23.

Novellis, V. De, Luongo, L., Guida, F., Cristino, L., Palazzo, E., Russo, R., et al. (2012). Effects of intra-ventrolateral periaqueductal grey palmitoylethanolamide on thermoceptive threshold and rostral ventromedial medulla cell activity. Eur. J. Pharmacol. *676*: 41–50.

Nozaki, C., Nagase, H., Nemoto, T., Matifas, A., Kieffer, B.L., and Gaveriaux-Ruff, C. (2014). In vivo properties of KNT-127, a novel delta opioid receptor agonist: receptor internalization, antihyperalgesia and antidepressant effects in mice. Br. J. Pharmacol. *171*: 5376–5386.

O' Mahony, S.M., Clarke, G., McKernan, D.P., Bravo, J.A., Dinan, T.G., and Cryan, J.F. (2013). Differential visceral nociceptive, behavioural and neurochemical responses to an immune challenge in the stress-sensitive Wistar Kyoto rat strain. Behav. Brain Res. *253*: 310–317.

O'Mahony, S.M., Bulmer, D.C., Coelho, A.-M., Fitzgerald, P., Bongiovanni, C., Lee, K., et al. (2010). 5-HT(2B) receptors modulate visceral hypersensitivity in a stress-sensitive animal model of brain-gut axis dysfunction. Neurogastroenterol. Motil. *22*: 573–8, e124.

O'Malley, D., Julio-Pieper, M., Gibney, S.M., Dinan, T.G., and Cryan, J.F. (2010). Distinct alterations in colonic morphology and physiology in two rat models of enhanced stress-induced anxiety and depression-like behaviour. Stress *13*: 114–122.

Ohayon, M.M., and Schatzberg, A.F. (2003). Using chronic pain to predict depressive

morbidity in the general population. Arch. Gen. Psychiatry *60*: 39–47.

Oka, T., Tsumori, T., Yokota, S., and Yasui, Y. (2008). Neuroanatomical and neurochemical organization of projections from the central amygdaloid nucleus to the nucleus retroambiguus via the periaqueductal gray in the rat. Neurosci. Res. *62*: 286–298.

Okamoto, K., and Aoki, K. (1963). Development of a strain of spontaneously hypertensive rats. Jpn. Circ. J. *27*: 282–293.

Okine, B.N., Gaspar, J.C., Madasu, M.K., Olango, W.M., Harhen, B., Roche, M., et al. (2017). Characterisation of peroxisome proliferator-activated receptor signalling in the midbrain periaqueductal grey of rats genetically prone to heightened stress, negative affect and hyperalgesia. Brain Res. *1657*: 185–192.

Olango, W.M., and Finn, D.P. (2014). Neurobiology of stress-induced hyperalgesia. Curr. Top. Behav. Neurosci. *20*: 251–280.

Olianas, M.C., Dedoni, S., and Onali, P. (2011). Regulation of PI3K/Akt signaling by N-desmethylclozapine through activation of δ-opioid receptor. Eur. J. Pharmacol. *660*: 341–350.

Omiya, Y., Goto, K., Ishige, A., and Komatsu, Y. (2000). Changes in Analgesia-Producing Mechanism of Repeated Cold Stress Loading in Mice. Pharmacol. Biochem. Behav. *65*: 261–266.

Ong, W.-Y., Stohler, C.S., and Herr, D.R. (2019). Role of the Prefrontal Cortex in Pain Processing. Mol. Neurobiol. *56*: 1137–1166.

Ortego, G., Villafañe, J.H., Doménech-García, V., Berjano, P., Bertozzi, L., and Herrero, P. (2016). Is there a relationship between psychological stress or anxiety and chronic nonspecific neck-arm pain in adults? A systematic review and meta-analysis. J. Psychosom. Res. *90*: 70–81.

Ossipov, M.H., Dussor, G.O., and Porreca, F. (2010). Central modulation of pain. J. Clin. Invest. *120*: 3779–87.

Ossipov, M.H., Morimura, K., and Porreca, F. (2014). Descending pain modulation and chronification of pain. Curr. Opin. Support. Palliat. Care *8*: 143–151.

Ozawa, A., Brunori, G., Mercatelli, D., Wu, J., Cippitelli, A., Zou, B., et al. (2015). Knock-In Mice with NOP-eGFP Receptors Identify Receptor Cellular and Regional Localization. J. Neurosci. *35*: 11682–11693.

Pais-Vieira, M., Lima, D., and Galhardo, V. (2009). Sustained attention deficits in rats with chronic inflammatory pain. Neurosci. Lett. *463*: 98–102.

Pardon, M.C., Gould, G.G., Garcia, A., Phillips, L., Cook, M.C., Miller, S.A., et al. (2002). Stress reactivity of the brain noradrenergic system in three rat strains differing in their neuroendocrine and behavioral responses to stress: Implications for susceptibility to stress-related neuropsychiatric disorders. Neuroscience *115*: 229–242.

Paré, D., Smith, Y., and Paré, J.F. (1995). Intra-amygdaloid projections of the basolateral and basomedial nuclei in the cat: Phaseolus vulgaris-leucoagglutinin anterograde tracing at the light and electron microscopic level. Neuroscience *69*: 567–583.

Paré, W.P. (1989a). Strain, age, but not gender, influence ulcer severity induced by water-restraint stress. Physiol. Behav. *45*: 627–632.

Paré, W.P. (1989b). Stress ulcer and open-field behavior of spontaneously hypertensive,

normotensive, and Wistar rats. Pavlov. J. Biol. Sci. *24*: 54–57.

Paré, W.P. (1989c). Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. Physiol. Behav. *46*: 993–998.

Paré, W.P. (1992). The performance of WKY rats on three tests of emotional behavior. Physiol. Behav. *51*: 1051–1056.

Paré, W.P. (1994). Open field, learned helplessness, conditioned defensive burying, and forced-swim tests in WKY rats. Physiol. Behav. *55*: 433–439.

Paré, W.P. (2000). Investigatory behavior of a novel conspecific by Wistar Kyoto, Wistar and Sprague-Dawley rats. Brain Res. Bull. *53*: 759–765.

Paré, W.P., and Kluczynski, J. (1997). Differences in the stress response of Wistar-Kyoto (WKY) rats from different vendors. Physiol. Behav. *62*: 643–648.

Paré, W.P., and Redei, E. (1993). Depressive behavior and stress ulcer in Wistar Kyoto rats. J. Physiol. - Paris *87*: 229–238.

Pare, W.P., and Tejani-Butt, S.M. (1996). Effect of stress on the behavior and 5-HT system in Sprague-Dawley and Wistar Kyoto rat strains. Integr. Physiol. Behav. Sci. *31*: 112–121.

Parent, A.J., Beaudet, N., Beaudry, H., Bergeron, J., Bérubé, P., Drolet, G., et al. (2012). Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain. Behav. Brain Res. *229*: 160–167.

Paretkar, T., and Dimitrov, E. (2019). Activation of enkephalinergic (Enk) interneurons in the central amygdala (CeA) buffers the behavioral effects of persistent pain. Neurobiol. Dis. *124*: 364–372.

Paris, J.J., Reilley, K.J., and McLaughlin, J.P. (2011). Kappa Opioid Receptor-Mediated Disruption of Novel Object Recognition: Relevance for Psychostimulant Treatment. J. Addict. Res. Ther. *S4*: 007.

Paxinos, G., and Watson, C. (2007). The rat brain in stereotaxic coordinates. (San Diego, USA: Academic Press).

Pearson, K.A., Stephen, A., Beck, S.G., and Valentino, R.J. (2006). Identifying genes in monoamine nuclei that may determine stress vulnerability and depressive behavior in Wistar-Kyoto rats. Neuropsychopharmacology *31*: 2449–2461.

Peckys, D., and Landwehrmeyer, G.B. (1999). Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: A 33P in situ hybridization study. Neuroscience *88*: 1093–1135.

Pellissier, L.P., Pujol, C.N., Becker, J.A.J., and Merrer, J. Le (2018). Delta Opioid Receptors: Learning and Motivation. Handb. Exp. Pharmacol. *247*: 227–260.

Peres, M.F.P., Mercante, J.P.P., Tobo, P.R., Kamei, H., and Bigal, M.E. (2017). Anxiety and depression symptoms and migraine: a symptom-based approach research. J. Headache Pain *18*: 37.

Pert, C.B., and Snyder, S.H. (1973). Properties of opiate-receptor binding in rat brain. Proc. Natl. Acad. Sci. U. S. A. *70*: 2243–2247.

Pertovaara, A. (1998). A neuronal correlate of secondary hyperalgesia in the rat spinal dorsal horn is submodality selective and facilitated by supraspinal influence. Exp. Neurol. *149*: 193–202.

Pfeiffer, A., Brantl, V., Herz, A., and Emrich, H.M. (1986). Psychotomimesis mediated by kappa opiate receptors. Science *233*: 774–776.

Piercey, M.F., and Einspahr, F.J. (1989). Spinal analgesic actions of kappa receptor agonists, U-50488H and spiradoline (U-62066). J. Pharmacol. Exp. Ther. *251*: 267–271.

Pilcher, C.W., and Browne, J.L. (1983). Effects of naloxone and Mr 1452 on stress-induced changes in nociception of different stimuli in rats. Life Sci. *33 Suppl 1*: 697–700.

Pitkänen, A., Savander, V., and LeDoux, J.E. (1997). Organization of intra-amygdaloid circuitries in the rat: An emerging framework for understanding functions of the amygdala. Trends Neurosci. *20*: 517–523.

Pitzer, C., Porta, C. La, Treede, R.-D., and Tappe-Theodor, A. (2019). Inflammatory and neuropathic pain conditions do not primarily evoke anxiety-like behaviours in C57BL/6 mice. Eur. J. Pain *23*: 285–306.

Plesan, A., Hoffmann, O., Xu, X.J., and Wiesenfeld-Hallin, Z. (1999). Genetic differences in the antinociceptive effect of morphine and its potentiation by dextromethorphan in rats. Neurosci. Lett. *263*: 53–56.

Polo, S., Diaz, A.F., Gallardo, N., Leanez, S., Balboni, G., and Pol, O. (2019). Treatment With the Delta Opioid Agonist UFP-512 Alleviates Chronic Inflammatory and Neuropathic Pain: Mechanisms Implicated. Front. Pharmacol. *10*: 283.

Pompili, M., Innamorati, M., Serafini, G., Gonda, X., Campi, S., Rapinesi, C., et al. (2012). How does subjective experience of pain relate to psychopathology among psychiatric patients? Gen. Hosp. Psychiatry *34*: 534–540.

Poole, H., White, S., Blake, C., Murphy, P., and Bramwell, R. (2009). Depression in chronic pain patients: prevalence and measurement. Pain Pract. *9*: 173–180.

Popa, S.L., Leucuta, D.C., and Dumitrascu, D.L. (2018). Pressure management as an occupational stress risk factor in irritable bowel syndrome: A cross-sectional study. Medicine (Baltimore). *97*: e13562.

Porreca, F., Mosberg, H.I., Omnaas, J.R., Burks, T.F., and Cowan, A. (1987). Supraspinal and spinal potency of selective opioid agonists in the mouse writhing test. J. Pharmacol. Exp. Ther. *240*: 890–894.

Porreca, F., Ossipov, M.H., and Gebhart, G.F. (2002). Chronic pain and medullary descending facilitation. Trends Neurosci. *25*: 319–325.

Porsolt, R.D., Brossard, G., Hautbois, C., and Roux, S. (2001). Rodent models of depression: forced swimming and tail suspension behavioral despair tests in rats and mice. Curr. Protoc. Neurosci. *14*: 8.10A.1-8.10A.10.

Pradhan, A., Smith, M., McGuire, B., Evans, C., and Walwyn, W. (2013). Chronic inflammatory injury results in increased coupling of delta opioid receptors to voltage-gated Ca2+ channels. Mol. Pain *9*: 8.

Pradhan, A.A., Befort, K., Nozaki, C., Gavériaux-Ruff, C., and Kieffer, B.L. (2011). The delta opioid receptor: An evolving target for the treatment of brain disorders. Trends Pharmacol. Sci. *32*: 581–590.

Pradhan, A.A.A., Walwyn, W., Nozaki, C., Filliol, D., Erbs, E., Matifas, A., et al. (2010). Ligand-directed trafficking of the δ-opioid receptor in vivo: Two paths toward analgesic tolerance. J. Neurosci. *30*: 16459–16468.

Presley, R.W., Menetrey, D., Levine, J.D., and Basbaum, A.I. (1990). Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J. Neurosci. *10*: 323–335.

Price, D.D. (2000). Psychological and neural mechanisms of the affective dimension of pain. Science *288*: 1769–1772.

Privette, T.H., and Terrian, D.M. (1995). Kappa opioid agonists produce anxiolytic-like behavior on the elevated plus-maze. Psychopharmacology (Berl). *118*: 444–450.

Puglisi-Allegra, S., Cabib, S., and Oliverio, A. (1986). Chronic stress reduces the analgesic but not the stimulant effect of morphine in mice. Brain Res. *380*: 357–358.

Qi, J., Chen, C., Lu, Y.-C., Zhang, T., Xu, H., Cui, Y.-Y., et al. (2014). Activation of extracellular signal-regulated kinase1/2 in the medial prefrontal cortex contributes to stress-induced hyperalgesia. Mol. Neurobiol. *50*: 1013–1023.

Qi, J., Chen, C., Meng, Q.-X., Wu, Y., Wu, H., and Zhao, T.-B. (2016). Crosstalk between Activated Microglia and Neurons in the Spinal Dorsal Horn Contributes to Stress-induced Hyperalgesia. Sci. Rep. *6*: 39442.

Qu, C., King, T., Okun, A., Lai, J., Fields, H.L., and Porreca, F. (2011). Lesion of the rostral anterior cingulate cortex eliminates the aversiveness of spontaneous neuropathic pain following partial or complete axotomy. Pain *152*: 1641–1648.

Quintero, L., Cardenas, R., and Suarez-Roca, H. (2011). Stress-induced hyperalgesia is associated with a reduced and delayed GABA inhibitory control that enhances post-synaptic NMDA receptor activation in the spinal cord. Pain *152*: 1909–1922.

Quintero, L., Cuesta, M.C., Silva, J.A., Arcaya, J.L., Pinerua-Suhaibar, L., Maixner, W., et al. (2003). Repeated swim stress increases pain-induced expression of c-Fos in the rat lumbar cord. Brain Res. *965*: 259–268.

Raftery, M.N., Ryan, P., Normand, C., Murphy, A.W., La Harpe, D. De, and McGuire, B.E. (2012). The economic cost of chronic noncancer pain in Ireland: Results from the PRIME study, part 2. J. Pain *13*: 139–145.

Raftery, M.N., Sarma, K., Murphy, A.W., la Harpe, D. De, Normand, C., and McGuire, B.E. (2011). Chronic pain in the Republic of Ireland--community prevalence, psychosocial profile and predictors of pain-related disability: results from the Prevalence, Impact and Cost of Chronic Pain (PRIME) study, part 1. Pain *152*: 1096–1103.

Rahman, W., Suzuki, R., Webber, M., Hunt, S.P., and Dickenson, A.H. (2006). Depletion of endogenous spinal 5-HT attenuates the behavioural hypersensitivity to mechanical and cooling stimuli induced by spinal nerve ligation. Pain *123*: 264–274.

Randic, M., Cheng, G., and Kojic, L. (1995). kappa-opioid receptor agonists modulate excitatory transmission in substantia gelatinosa neurons of the rat spinal cord. J. Neurosci. *15*: 6809–6826.

Rasakham, K., and Liu-Chen, L.-Y. (2011). Sex differences in kappa opioid pharmacology. Life Sci. *88*: 2–16.

Rashvand, M., Khajavai, A., Parviz, M., Hasanein, P., and Keshavarz, M. (2014). GABAA receptors are involved in the analgesic effects of morphine microinjected into the central nucleus of the amygdala. Clin. Exp. Pharmacol. Physiol. *41*: 338–344.

Raynor, K., Kong, H., Chen, Y., Yasuda, K., Yu, L., Bell, G.I., et al. (1994). Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors.

Mol. Pharmacol. *45*: 330–334.

Rea, K., Olango, W.M., Okine, B.N., Madasu, M.K., McGuire, I.C., Coyle, K., et al. (2014). Impaired endocannabinoid signalling in the rostral ventromedial medulla underpins genotype-dependent hyper-responsivity to noxious stimuli. Pain *155*: 69–79.

Redei, E., Pare, W.P., Aird, F., and Kluczynski, J. (1994). Strain differences in hypothalamic-pituitary-adrenal activity and stress ulcer. Am. J. Physiol. *266*: R353-60.

Reed, B., Butelman, E.R., Fry, R.S., Kimani, R., and Kreek, M.J. (2018). Repeated Administration of Opra Kappa (LY2456302), a Novel, Short-Acting, Selective KOP-r Antagonist, in Persons with and without Cocaine Dependence. Neuropsychopharmacology *43*: 739–750.

Refsgaard, L.K., Hoffmann-Petersen, J., Sahlholt, M., Pickering, D.S., and Andreasen, J.T. (2016). Modelling affective pain in mice: Effects of inflammatory hypersensitivity on place escape/avoidance behaviour, anxiety and hedonic state. J. Neurosci. Methods *262*: 85–92.

Reid, K.J., Harker, J., Bala, M.M., Truyers, C., Kellen, E., Bekkering, G.E., et al. (2011). Epidemiology of chronic non-cancer pain in Europe: narrative review of prevalence, pain treatments and pain impact. Curr. Med. Res. Opin. *27*: 449–462.

Reindl, J.D., Rowan, K., Carey, A.N., Peng, X., Neumeyer, J.L., and McLaughlin, J.P. (2008). Antidepressant-like effects of the novel kappa opioid antagonist MCL-144B in the forced-swim test. Pharmacology *81*: 229–235.

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., et al. (1995). Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science *270*: 792–794.

Renno, W.M., Mahmoud, M.S., Hamdi, A., and Beitz, A.J. (1999). Quantitative immunoelectron microscopic colocalization of GABA and enkephalin in the ventrocaudal periaqueductal gray of the rat. Synapse *31*: 216–228.

Reul, J.M.H.., and Holsboer, F. (2002). Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr. Opin. Pharmacol. *2*: 23–33.

Reynolds, D. V (1969). Surgery in the rat during electrical analgesia induced by focal brain stimulation. Science *164*: 444–445.

Reynolds, J., Bilsky, E.J., and Meng, I.D. (2011). Selective ablation of mu-opioid receptor expressing neurons in the rostral ventromedial medulla attenuates stress-induced mechanical hypersensitivity. Life Sci. *89*: 313–319.

Rhudy, J.L., and Meagher, M.W. (2000). Fear and anxiety: divergent effects on human pain thresholds. Pain *84*: 65–75.

Rimoy, G.H., Wright, D.M., Bhaskar, N.K., and Rubin, P.C. (1994). The cardiovascular and central nervous system effects in the human of U-62066E. A selective opioid receptor agonist. Eur. J. Clin. Pharmacol. *46*: 203–207.

Rittenhouse, P.A., López-Rubalcava, C., Stanwood, G.D., and Lucki, I. (2002). Amplified behavioral and endocrine responses to forced swim stress in the Wistar-Kyoto rat. Psychoneuroendocrinology *27*: 303–318.

Rivat, C., Becker, C., Blugeot, A., Zeau, B., Mauborgne, A., Pohl, M., et al. (2010). Chronic stress induces transient spinal neuroinflammation, triggering sensory hypersensitivity and long-lasting anxiety-induced hyperalgesia. Pain *150*: 358–368.

Rizvi, T.A., Ennis, M., Behbehani, M.M., and Shipley, M.T. (1991). Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity. J. Comp. Neurol. *303*: 121–31.

Robbins, M.T., DeBerry, J., and Ness, T.J. (2007). Chronic psychological stress enhances nociceptive processing in the urinary bladder in high-anxiety rats. Physiol. Behav. *91*: 544–550.

Robles, C.F., McMackin, M.Z., Campi, K.L., Doig, I.E., Takahashi, E.Y., Pride, M.C., et al. (2014). Effects of kappa opioid receptors on conditioned place aversion and social interaction in males and females. Behav. Brain Res. *262*: 84–93.

Roeckel, L.A., Coz, G.M. Le, Gavériaux-Ruff, C., and Simonin, F. (2016). Opioid-induced hyperalgesia: Cellular and molecular mechanisms. Neuroscience *338*: 160–182.

Roeska, K., Doods, H., Arndt, K., Treede, R.-D., and Ceci, A. (2008). Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin. Pain *139*: 349–357.

Rorick-Kehn, L.M., Witkin, J.M., Statnick, M.A., Eberle, E.L., McKinzie, J.H., Kahl, S.D., et al. (2014). LY2456302 is a novel, potent, orally-bioavailable small molecule kappa-selective antagonist with activity in animal models predictive of efficacy in mood and addictive disorders. Neuropharmacology *77*: 131–144.

Rosa, P.B., Ribeiro, C.M., Bettio, L.E.B., Colla, A., Lieberknecht, V., Moretti, M., et al. (2014). Folic acid prevents depressive-like behavior induced by chronic corticosterone treatment in mice. Pharmacol. Biochem. Behav. *127*: 1–6.

Rosa, S.G., Pesarico, A.P., Tagliapietra, C.F., Luz, S.C. da, and Nogueira, C.W. (2017). Opioid system contribution to the antidepressant-like action of m-trifluoromethyl-diphenyl diselenide in mice: A compound devoid of tolerance and withdrawal syndrome. J. Psychopharmacol. *31*: 1250–1262.

Rosenberger, C., Elsenbruch, S., Scholle, A., Greiff, A. de, Schedlowski, M., Forsting, M., et al. (2009). Effects of psychological stress on the cerebral processing of visceral stimuli in healthy women. Neurogastroenterol. Motil. *21*: 740-e45.

Rosenthal, S. (1949). Histamine as possible chemical mediator for cutaneous pain; painful responses on intradermal injection of perfusates from stimulated human skin. J. Appl. Physiol. *2*: 348–354.

Rossi, G., Pan, Y.X., Cheng, J., and Pasternak, G.W. (1994a). Blockade of morphine analgesia by an antisense oligodeoxynucleotide against the mu receptor. Life Sci. *54*: PL375–PL379.

Rossi, G.C., Pasternak, G.W., and Bodnar, R.J. (1994b). μ and δ opioid synergy between the periaqueductal gray and the rostro-ventral medulla. Brain Res. *665*: 85–93.

Roy, C. Le, Laboureyras, E., Gavello-Baudy, S., Chateauraynaud, J., Laulin, J.P., and Simonnet, G. (2011). Endogenous opioids released during non-nociceptive environmental stress induce latent pain sensitization via a NMDA-dependent process. J. Pain *12*: 1069–1079.

Ruda, M.A., Allen, B., and Gobel, S. (1981). Ultrastructural analysis of medial brain stem afferents to the superficial dorsal horn. Brain Res. *205*: 175–180.

Rusin, K.I., Giovannucci, D.R., Stuenkel, E.L., and Moises, H.C. (1997). Kappa-opioid receptor activation modulates Ca2+ currents and secretion in isolated neuroendocrine

nerve terminals. J. Neurosci. *17*: 6565–6574.

Russo, R., LoVerme, J., Rana, G. La, D'Agostino, G., Sasso, O., Calignano, A., et al. (2007). Synergistic antinociception by the cannabinoid receptor agonist anandamide and the PPAR-α receptor agonist GW7647. Eur. J. Pharmacol. *566*: 117–119.

Ruzicka, B.B., Fox, C.A., Thompson, R.C., Meng, F., Watson, S.J., and Akil, H. (1995). Primary astroglial cultures derived from several rat brain regions differentially express mu, delta and kappa opioid receptor mRNA. Brain Res. Mol. Brain Res. *34*: 209–220.

Saarialho-Kere, U., Mattila, M.J., and Seppala, T. (1989). Psychomotor, respiratory and neuroendocrinological effects of a mu-opioid receptor agonist (oxycodone) in healthy volunteers. Pharmacol. Toxicol. *65*: 252–257.

Sabetkasaei, M., Masoudnia, F., Khansefid, N., and Behzadi, G. (2007). Opioid receptors of the central amygdala and morphine-induced antinociception. Iran. Biomed. J. *11*: 75–80.

Sadeghi, M., Peeri, M., and Hosseini, M.J. (2016). Adolescent voluntary exercise attenuated hippocampal innate immunity responses and depressive-like behaviors following maternal separation stress in male rats. Physiol. Behav. *163*: 177–183.

Sagvolden, T., Dasbanerjee, T., Zhang-James, Y., Middleton, F., and Faraone, S. (2008). Behavioral and genetic evidence for a novel animal model of Attention-Deficit/Hyperactivity Disorder Predominantly Inattentive Subtype. Behav. Brain Funct. *4*: 56.

Saitoh, A., Kimura, Y., Suzuki, T., Kawai, K., Nagase, H., and Kamei, J. (2004). Potential anxiolytic and antidepressant-like activities of SNC80, a selective δ-opioid agonist, in behavioral models in rodents. J. Pharmacol. Sci. *95*: 374–380.

Saitoh, A., Sugiyama, A., Nemoto, T., Fujii, H., Wada, K., Oka, J.I., et al. (2011). The novel δ opioid receptor agonist KNT-127 produces antidepressant-like and antinociceptive effects in mice without producing convulsions. Behav. Brain Res. *223*: 271–279.

Saitoh, A., Yamada, M., Yamada, M., Takahashi, K., Yamaguchi, K., Murasawa, H., et al. (2008). Antidepressant-like effects of the delta-opioid receptor agonist SNC80 ([(+)-4-[(alphaR)-alpha-[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-methoxyphenyl)methyl]-N,N-diethylbenzamide) in an olfactory bulbectomized rat model. Brain Res. *1208*: 160–169.

Salemi, S., Aeschlimann, A., Reisch, N., Jungel, A., Gay, R.E., Heppner, F.L., et al. (2005). Detection of kappa and delta opioid receptors in skin--outside the nervous system. Biochem. Biophys. Res. Commun. *338*: 1012–1017.

Sandin, J., Nylander, I., Georgieva, J., Schött, P.A., Ögren, S.O., and Terenius, L. (1998). Hippocampal dynorphin B injections impair spatial learning in rats: A κ-opioid receptor-mediated effect. Neuroscience *85*: 375–382.

Sanoja, R., Tortorici, V., Fernandez, C., Price, T.J., and Cervero, F. (2010). Role of RVM neurons in capsaicin-evoked visceral nociception and referred hyperalgesia. Eur. J. Pain *14*: 120.e1–9.

Sante, A.B., Nobre, M.J., and Brandao, M.L. (2000). Place aversion induced by blockade of mu or activation of kappa opioid receptors in the dorsal periaqueductal gray matter. Behav. Pharmacol. *11*: 583–589.

Sardi, N.F., Lazzarim, M.K., Guilhen, V.A., Marcílio, R.S., Natume, P.S., Watanabe, T.C., et al. (2018). Chronic sleep restriction increases pain sensitivity over time in a periaqueductal gray and nucleus accumbens dependent manner. Neuropharmacology *139*: 52–60.

Sarton, E., Olofsen, E., Romberg, R., Hartigh, J. den, Kest, B., Nieuwenhuijs, D., et al. (2000). Sex differences in morphine analgesia: an experimental study in healthy volunteers. Anesthesiology *93*: 1245–54; discussion 6A.

Satoh, M., Kubota, A., Iwama, T., Wada, T., Yasui, M., Fujibayashi, K., et al. (1983). Comparison of analgesic potencies of mu, delta and kappa agonists locally applied to various CNS regions relevant to analgesia in rats. Life Sci. *33*: 689–692.

Satyanarayanan, S.K., Shih, Y.-H., Wen, Y.-R., Palani, M., Lin, Y.-W., Su, H., et al. (2019). miR-200a-3p modulates gene expression in comorbid pain and depression: Molecular implication for central sensitization. Brain. Behav. Immun. *82*: 230–238.

Sawada, A., Niiyama, Y., Ataka, K., Nagaishi, K., Yamakage, M., and Fujimiya, M. (2014). Suppression of bone marrow-derived microglia in the amygdala improves anxiety-like behavior induced by chronic partial sciatic nerve ligation in mice. Pain *155*: 1762–1772.

Scherrer, G., Tryoen-Toth, P., Filliol, D., Matifas, A., Laustriat, D., Cao, Y.Q., et al. (2006). Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. Proc. Natl. Acad. Sci. U. S. A. *103*: 9691–9696.

Schlen, H., and Bentley, G.A. (1980). The possibility that a component of morphine-induced analgesia is contributed indirectly via the release of endogenous opioids. Pain *9*: 73–84.

Schmidhammer, H., Burkard, W., Eggstein-Aeppli, L., and Smith, C. (1989). Synthesis and biological evaluation of 14-alkoxymorphinans. 2. (-)-N-(cyclopropylmethyl)-4,14-dimethoxymorphinan-6-one, a selective mu opioid receptor antagonist. J. Med. Chem. *32*: 418–21.

Schmidt-Wilcke, T., Leinisch, E., Straube, A., Kampfe, N., Draganski, B., Diener, H.C., et al. (2005). Gray matter decrease in patients with chronic tension type headache. Neurology *65*: 1483–1486.

Schmitz, R. (1985). Friedrich Wilhelm Serturner and the discovery of morphine. Pharm. Hist. *27*: 61–74.

Scholl, J.L., Renner, K.J., Forster, G.L., and Tejani-Butt, S. (2010). Central monoamine levels differ between rat strains used in studies of depressive behavior. Brain Res. *1355*: 41–51.

Schrader, A.J., Taylor, R.M., Lowery-Gionta, E.G., and Moore, N.L.T. (2018). Repeated elevated plus maze trials as a measure for tracking within-subjects behavioral performance in rats (Rattus norvegicus). PLoS One *13*: e0207804.

Schramm, S.H., Moebus, S., Lehmann, N., Galli, U., Obermann, M., Bock, E., et al. (2015). The association between stress and headache: A longitudinal population-based study. Cephalalgia *35*: 853–863.

Schwartz, J.C., Malfroy, B., and La Baume, S. De (1981). Biological inactivation of enkephalins and the role of enkephalin-dipeptidyl-carboxypeptidase (‘enkephalinase’) as neuropeptidase. Life Sci. *29*: 1715–1740.

Schwartz, R.W., Chang, A.C., Portoghese, P.S., and Berzetei-Gurske, I.P. (1997). A guinea pig ileum preparation devoid of functional κ receptors: A new in vitro pharmacologic assay for μ-opioid ligands. Life Sci. *60*: PL235–PL239.

Scott, E.L., Kroenke, K., Wu, J., and Yu, Z. (2016). Beneficial effects of improvement in depression, pain catastrophizing, and anxiety on pain outcomes: A 12-month longitudinal analysis. J. Pain *17*: 215–222.

Seminowicz, D.A., Laferriere, A.L., Millecamps, M., Yu, J.S.C., Coderre, T.J., and Bushnell, M.C. (2009). MRI structural brain changes associated with sensory and emotional function in a rat model of long-term neuropathic pain. Neuroimage *47*: 1007–1014.

Sens, J., Schneider, E., Mauch, J., Schaffstein, A., Mohamed, S., Fasoli, K., et al. (2017). Lipopolysaccharide administration induces sex-dependent behavioural and serotonergic neurochemical signatures in mice. Pharmacol. Biochem. Behav. *153*: 168–181.

Seward, E., Hammond, C., and Henderson, G. (1991). Mu-opioid-receptor-mediated inhibition of the N-type calcium-channel current. Proceedings. Biol. Sci. *244*: 129–135.

Sharpe, L.G., Garnett, J.E., and Cicero, T.J. (1974). Analgesia and hyperreactivity produced by intracranial microinjections of morphine into the periaqueductal gray matter of the rat. Behav. Biol. *11*: 303–313.

Sheahan, T.D., Siuda, E.R., Bruchas, M.R., Shepherd, A.J., Mohapatra, D.P., Gereau, R.W., et al. (2017). Inflammation and nerve injury minimally affect mouse voluntary behaviors proposed as indicators of pain. Neurobiol. Pain *2*: 1–12.

Shetty, R.A., and Sadananda, M. (2017). Brief Social Isolation in the Adolescent Wistar-Kyoto Rat Model of Endogenous Depression Alters Corticosterone and Regional Monoamine Concentrations. Neurochem. Res. *42*: 1470–1477.

Shi, C., and Davis, M. (1999). Pain pathways involved in fear conditioning measured with fear-potentiated startle: lesion studies. J. Neurosci. *19*: 420–430.

Shi, M., Wang, J.-Y.Y., and Luo, F. (2010). Depression shows divergent effects on evoked and spontaneous pain behaviors in rats. J. Pain *11*: 219–229.

Shin, I.C., Kim, H.C., Swanson, J., Hong, J.T., and Oh, K.W. (2003). Anxiolytic effects of acute morphine can be modulated by nitric oxide systems. Pharmacology *68*: 183–189.

Shin, S.Y., Katz, P., Wallhagen, M., and Julian, L. (2012). Cognitive impairment in persons with rheumatoid arthritis. Arthritis Care Res. (Hoboken). *64*: 1144–1150.

Shippenberg, T.S., and Herz, A. (1987). Place preference conditioning reveals the involvement of D1-dopamine receptors in the motivational properties of μ- and κ-opioid agonists. Brain Res. *436*: 169–172.

Shirayama, Y., Ishida, H., Iwata, M., Hazama, G.-I., Kawahara, R., and Duman, R.S. (2004). Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects. J. Neurochem. *90*: 1258–1268.

Shoval, G., Shbiro, L., Hershkovitz, L., Hazut, N., Zalsman, G., Mechoulam, R., et al. (2016). Prohedonic effect of cannabidiol in a rat model of depression. Neuropsychobiology *73*: 123–129.

Sieberg, C.B., Taras, C., Gomaa, A., Nickerson, C., Wong, C., Ward, C., et al. (2018). Neuropathic pain drives anxiety behavior in mice, results consistent with anxiety levels

in diabetic neuropathy patients. Pain Reports *3*: e651.

Silva Torres, I.L. da, Cucco, S.N.S., Bassani, M., Duarte, M.S., Silveira, P.P., Vasconcellos, A.P., et al. (2003). Long-lasting delayed hyperalgesia after chronic restraint stress in rats-effect of morphine administration. Neurosci. Res. *45*: 277–283.

Simantov, R., and Snyder, S.H. (1976). Morphine-like peptides in mammalian brain: isolation, structure elucidation, and interactions with the opiate receptor. Proc. Natl. Acad. Sci. U. S. A. *73*: 2515–2519.

Simonin, F., Gaveriaux-Ruff, C., Befort, K., Matthes, H., Lannes, B., Micheletti, G., et al. (1995). kappa-Opioid receptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system. Proc. Natl. Acad. Sci. U. S. A. *92*: 7006–7010.

Simonin, F., Kieffer, B.L., Valverde, O., Smadja, C., Roques, B.P., Maldonado, R., et al. (1998). Disruption of the κ-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective κ-agonist U-50,488H and attenuates morphine withdrawal. EMBO J. *17*: 886–897.

Simons, L.E., Elman, I., and Borsook, D. (2014a). Psychological processing in chronic pain: A neural systems approach. Neurosci. Biobehav. Rev. *39*: 61–78.

Simons, L.E., Moulton, E.A., Linnman, C., Carpino, E., Becerra, L., and Borsook, D. (2014b). The human amygdala and pain: evidence from neuroimaging. Hum. Brain Mapp. *35*: 527–538.

Sitsen, J.M., Nijkamp, F.P., and Jong, W. de (1987). Differential sensitivity to morphine in spontaneously hypertensive and normotensive Wistar-Kyoto and Wistar rats. Clin. Exp. Hypertens. A. *9*: 1159–1171.

Slattery, D.A., and Cryan, J.F. (2012). Using the rat forced swim test to assess antidepressant-like activity in rodents. Nat. Protoc. *7*: 1009–1014.

Smith, D.J., Perrotti, J.M., Crisp, T., Cabral, M.E.Y., Long, J.T., and Scalzitti, J.M. (1988). The μ opiate receptor is responsible for descending pain inhibition originating in the periaqueductal gray region of the rat brain. Eur. J. Pharmacol. *156*: 47–54.

Smith, J.S., Schindler, A.G., Martinelli, E., Gustin, R.M., Bruchas, M.R., and Chavkin, C. (2012). Stress-induced activation of the dynorphin/kappa-opioid receptor system in the amygdala potentiates nicotine conditioned place preference. J. Neurosci. *32*: 1488–1495.

Snyder, L.M., Chiang, M.C., Loeza-Alcocer, E., Omori, Y., Hachisuka, J., Sheahan, T.D., et al. (2018). Kappa Opioid Receptor Distribution and Function in Primary Afferents. Neuron *99*: 1274-1288.e6.

Soignier, R.D., Taylor, B.K., Baiamonte, B.A., Lee, F.A., Paul, D., and Gould, H.J. 3rd (2011). Measurement of CFA-induced hyperalgesia and morphine-induced analgesia in rats: dorsal vs plantar mechanical stimulation of the hindpaw. Pain Med. *12*: 451–458.

Sorge, R.E., and Totsch, S.K. (2017). Sex Differences in Pain. J. Neurosci. Res. *95*: 1271–1281.

Stander, S., Gunzer, M., Metze, D., Luger, T., and Steinhoff, M. (2002). Localization of mu-opioid receptor 1A on sensory nerve fibers in human skin. Regul. Pept. *110*: 75–83.

Stein, C., Millan, M.J., and Herz, A. (1988). Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. Pharmacol. Biochem. Behav. *31*: 445–451.

Stern, P., Nikulin, A., and Ferluga, J. (1962). The role of histamine and bradykinin in the inflammatory process. Arch. Int. Pharmacodyn. Ther. *140*: 528–38.

Stewart, P.E., and Hammond, D.L. (1994). Activation of spinal delta-1 or delta-2 opioid receptors reduces carrageenan-induced hyperalgesia in the rat. J. Pharmacol. Exp. Ther. *268*: 701–708.

Stoffel, E.C., Ulibarri, C.M., Folk, J.E., Rice, K.C., and Craft, R.M. (2005). Gonadal hormone modulation of mu, kappa, and delta opioid antinociception in male and female rats. J. Pain *6*: 261–274.

Stubbs, B., Aluko, Y., Myint, P.K., and Smith, T.O. (2016). Prevalence of depressive symptoms and anxiety in osteoarthritis: a systematic review and meta-analysis. Age Ageing *45*: 228–235.

Su, J., Tanaka, Y., Muratsubaki, T., Kano, M., Kanazawa, M., and Fukudo, S. (2015). Injection of corticotropin-releasing hormone into the amygdala aggravates visceral nociception and induces noradrenaline release in rats. Neurogastroenterol. Motil. *27*: 30–39.

Su, Y.-L., Wang, N., Gao, G., Wang, J.-Y., and Luo, F. (2010). The effect of depression on the thermal nociceptive thresholds in rats with spontaneous pain. Neurosci. Bull. *26*: 429–436.

Suarez-Roca, H., Quintero, L., Avila, R., Medina, S., Freitas, M. De, and Cárdenas, R. (2014). Central immune overactivation in the presence of reduced plasma corticosterone contributes to swim stress-induced hyperalgesia. Brain Res. Bull. *100*: 61–69.

Suarez-Roca, H., Silva, J.A., Arcaya, J.L., Quintero, L., Maixner, W., and Pinerua-Shuhaibar, L. (2006). Role of mu-opioid and NMDA receptors in the development and maintenance of repeated swim stress-induced thermal hyperalgesia. Behav. Brain Res. *167*: 205–211.

Sun, R., Zhang, W., Bo, J., Zhang, Z., Lei, Y., Huo, W., et al. (2017). Spinal activation of alpha7-nicotinic acetylcholine receptor attenuates posttraumatic stress disorder-related chronic pain via suppression of glial activation. Neuroscience *344*: 243–254.

Sun, R., Zhang, Z., Lei, Y., Liu, Y., Lu, C., Rong, H., et al. (2016a). Hippocampal activation of microglia may underlie the shared neurobiology of comorbid posttraumatic stress disorder and chronic pain. Mol. Pain *12*:.

Sun, T., Wang, J.-B., Li, X., Li, Y.J., Feng, D., Shi, W.L., et al. (2016b). Gastrodin relieved complete Freund's adjuvant-induced spontaneous pain by inhibiting inflammatory response. Int. Immunopharmacol. *41*: 66–73.

Suzuki, T., Amata, M., Sakaue, G., Nishimura, S., Inoue, T., Shibata, M., et al. (2007). Experimental neuropathy in mice is associated with delayed behavioral changes related to anxiety and depression. Anesth. Analg. *104*: 1570–7, table of contents.

Swett, J.E., and Woolf, C.J. (1985). The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. J. Comp. Neurol. *231*: 66–77.

Takasusuki, T., Yamaguchi, S., Hamaguchi, S., and Yaksh, T.L. (2013). Effects of general anesthetics on substance P release and c-Fos expression in the spinal dorsal horn. Anesthesiology *119*: 433–442.

Taloyan, M., and Lofvander, M. (2014). Depression and gender differences among

younger immigrant patients on sick leave due to chronic back pain: a primary care study. Prim. Health Care Res. Dev. *15*: 5–14.

Tanimoto, S., Nakagawa, T., Yamauchi, Y., Minami, M., and Satoh, M. (2003). Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. Eur. J. Neurosci. *18*: 2343–2350.

Tao, R., and Auerbach, S.B. (1994). Increased extracellular serotonin in rat brain after systemic or intraraphe administration of morphine. J. Neurochem. *63*: 517–524.

Tao, R., and Auerbach, S.B. (2005). μ-Opioids disinhibit and κ-opioids inhibit serotonin efflux in the dorsal raphe nucleus. Brain Res. *1049*: 70–79.

Taussig, R., Iniguez-Lluhi, J.A., and Gilman, A.G. (1993). Inhibition of adenylyl cyclase by Gi alpha. Science *261*: 218–221.

Taylor, B.K., and Basbaum, A.I. (2003). Systemic morphine-induced release of serotonin in the rostroventral medulla is not mimicked by morphine microinjection into the periaqueductal gray. J. Neurochem. *86*: 1129–1141.

Taylor, B.K., Roderick, R.E., St Lezin, E., and Basbaum, A.I. (2001). Hypoalgesia and hyperalgesia with inherited hypertension in the rat. Am. J. Physiol. Regul. Integr. Comp. Physiol. *280*: R345-54.

Tejani-Butt, S., Kluczynski, J., and Paré, W.P. (2003). Strain-dependent modification of behavior following antidepressant treatment. Prog. Neuro-Psychopharmacology Biol. Psychiatry *27*: 7–14.

Tejani-Butt, S.M., Paré, W.P., and Yang, J. (1994). Effect of repeated novel stressors on depressive behavior and brain norepinephrine receptor system in Sprague-Dawley and Wistar Kyoto (WKY) rats. Brain Res. *649*: 27–35.

Tejeda, H.A., and Bonci, A. (2019). Dynorphin/kappa-opioid receptor control of dopamine dynamics: Implications for negative affective states and psychiatric disorders. Brain Res. *1713*: 91–101.

Tejeda, H.A., Counotte, D.S., Oh, E., Ramamoorthy, S., Schultz-Kuszak, K.N., Backman, C.M., et al. (2013). Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion. Neuropsychopharmacology *38*: 1770–1779.

Tempel, A., and Zukin, R.S. (1987). Neuroanatomical patterns of the mu, delta, and kappa opioid receptors of rat brain as determined by quantitative in vitro autoradiography. Proc. Natl. Acad. Sci. U. S. A. *84*: 4308–4312.

Terayama, R., Guan, Y., Dubner, R., and Ren, K. (2000). Activity-induced plasticity in brain stem pain modulatory circuitry after inflammation. Neuroreport *11*: 1915–1919.

Terner, J.M., Lomas, L.M., Lewis, J.W., Husbands, S.M., and Picker, M.J. (2005). Effects of the long-lasting kappa opioid 2-(3,4-dichlorophenyl)-N-methyl-N- [(1S)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl) ethyl] acetamide in a drug discrimination and warm water tail-withdrawal procedure. Behav. Pharmacol. *16*: 665–670.

Terner, J.M., Lomas, L.M., Smith, E.S., Barrett, A.C., and Picker, M.J. (2003). Pharmacogenetic analysis of sex differences in opioid antinociception in rats. Pain *106*: 381–391.

Thompson, A.A., Liu, W., Chun, E., Katritch, V., Wu, H., Vardy, E., et al. (2012).

Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. Nature *485*: 395–399.

Thompson, S.J., Pitcher, M.H., Stone, L.S., Tarum, F., Niu, G., Chen, X., et al. (2018). Chronic neuropathic pain reduces opioid receptor availability with associated anhedonia in rat. Pain *159*: 1856–1866.

Tizabi, Y., Bhatti, B.H., Manaye, K.F., Das, J.R., and Akinfiresoye, L. (2012). Antidepressant-like effects of low ketamine dose is associated with increased hippocampal AMPA/NMDA receptor density ratio in female Wistar-Kyoto rats. Neuroscience *213*: 72–80.

Todtenkopf, M.S., Marcus, J.F., Portoghese, P.S., and Carlezon, W.A.J. (2004). Effects of kappa-opioid receptor ligands on intracranial self-stimulation in rats. Psychopharmacology (Berl). *172*: 463–470.

Toll, L., Berzetei-Gurske, I.P., Polgar, W.E., Brandt, S.R., Adapa, I.D., Rodriguez, L., et al. (1998). Standard binding and functional assays related to medications development division testing for potential cocaine and opiate narcotic treatment medications. NIDA Res. Monogr. *178*: 440–466.

Tomim, D.H., Pontarolla, F.M., Bertolini, J.F., Arase, M., Tobaldini, G., Lima, M.M.S., et al. (2016). The pronociceptive effect of paradoxical sleep deprivation in rats: evidence for a role of descending pain modulation mechanisms. Mol. Neurobiol. *53*: 1706–1717.

Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., et al. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron *21*: 531–543.

Torregrossa, M.M., Jutkiewicz, E.M., Mosberg, H.I., Balboni, G., Watson, S.J., and Woods, J.H. (2006). Peptidic delta opioid receptor agonists produce antidepressant-like effects in the forced swim test and regulate BDNF mRNA expression in rats. Brain Res. *1069*: 172–181.

Torsney, C., and MacDermott, A.B. (2006). Disinhibition opens the gate to pathological pain signaling in superficial neurokinin 1 receptor-expressing neurons in rat spinal cord. J. Neurosci. *26*: 1833–1843.

Tortorici, V., and Morgan, M.M. (2002). Comparison of morphine and kainic acid microinjections into identical PAG sites on the activity of RVM neurons. J. Neurophysiol. *88*: 1707–1715.

Tracey, I., and Mantyh, P.W. (2007). The Cerebral Signature for Pain Perception and Its Modulation. Neuron *55*: 377–391.

Treede, R.-D., Rief, W., Barke, A., Aziz, Q., Bennett, M.I., Benoliel, R., et al. (2019). Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). Pain *160*: 19–27.

Tsang, A., Korff, M. Von, Lee, S., Alonso, J., Karam, E., Angermeyer, M.C., et al. (2008). Common chronic pain conditions in developed and developing countries: gender and age differences and comorbidity with depression-anxiety disorders. J. Pain *9*: 883–891.

Tsang, S.W., Zhao, M., Wu, J., Sung, J.J.Y., and Bian, Z.-X. (2012). Nerve growth factor-mediated neuronal plasticity in spinal cord contributes to neonatal maternal separation-induced visceral hypersensitivity in rats. Eur. J. Pain *16*: 463–472.

Tsou, K., and Jang, C.S. (1964). Studies on the site of analgesic action of morphine by

intracerebral microinjection. Sci. Sin. *13*: 1099–109.

Tsukahara-Ohsumi, Y., Tsuji, F., Niwa, M., Hata, T., Narita, M., Suzuki, T., et al. (2011). The kappa opioid receptor agonist SA14867 has antinociceptive and weak sedative effects in models of acute and chronic pain. Eur. J. Pharmacol. *671*: 53–60.

Tuominen, L., Salo, J., Hirvonen, J., Någren, K., Laine, P., Melartin, T., et al. (2012). Temperament trait Harm Avoidance associates with μ-opioid receptor availability in frontal cortex: A PET study using [11C]carfentanil. Neuroimage *61*: 670–676.

Turner, B.H., and Herkenham, M. (1991). Thalamoamygdaloid projections in the rat: a test of the amygdala's role in sensory processing. J. Comp. Neurol. *313*: 295–325.

Twillman, R. (2008). Mental Disorders in Chronic Pain Patients. J. Pain Palliat. Care Pharmacother. *21*: 13–19.

Tzschentke, T.M. (1998). Measuring reward with the conditioned place preference paradigm: A comprehensive review of drug effects, recent progress and new issues. Prog. Neurobiol. *56*: 613–672.

Uhelski, M.L., and Fuchs, P.N. (2009). Naltrexone fails to increase pain affect in response to inflammatory pain in a novel escape/avoidance paradigm. Physiol. Behav. *98*: 263–267.

Uher, R., Payne, J.L., Pavlova, B., and Perlis, R.H. (2014). Major depressive disorder in DSM-5: implications for clinical practice and research of changes from DSM-IV. Depress. Anxiety *31*: 459–471.

Ukai, M., and Kameyama, T. (1985). Multi-dimensional analyses of behavior in mice treated with U-50,488H, a purported kappa (non-mu) opioid agonist. Brain Res. *337*: 352–356.

Urban, M.O., Jiang, M.C., and Gebhart, G.F. (1996). Participation of central descending nociceptive facilitatory systems in secondary hyperalgesia produced by mustard oil. Brain Res. *737*: 83–91.

Urban, M.O., and Smith, D.J. (1994). Nuclei within the rostral ventromedial medulla mediating morphine antinociception from the periaqueductal gray. Brain Res. *652*: 9–16.

Urban, R., Scherrer, G., Goulding, E.H., Tecott, L.H., and Basbaum, A.I. (2011). Behavioral indices of ongoing pain are largely unchanged in male mice with tissue or nerve injury-induced mechanical hypersensitivity. Pain *152*: 990–1000.

Urigüen, L., Fernández, B., Romero, E.M., Pedro, N. De, Delgado, M.J., Guaza, C., et al. (2002). Effects of 14-methoxymetopon, a potent opioid agonist, on the responses to the tail electric stimulation test and plus-maze activity in male rats: Neuroendocrine correlates. Brain Res. Bull. *57*: 661–666.

Vachon-Presseau, E., Roy, M., Martel, M.-O., Caron, E., Marin, M.-F., Chen, J., et al. (2013). The stress model of chronic pain: evidence from basal cortisol and hippocampal structure and function in humans. Brain *136*: 815–827.

Vachon-Presseau, E., Tetreault, P., Petre, B., Huang, L., Berger, S.E., Torbey, S., et al. (2016). Corticolimbic anatomical characteristics predetermine risk for chronic pain. Brain *139*: 1958–1970.

Valdez, G.R., and Harshberger, E. (2012). Kappa opioid regulation of anxiety-like behavior during acute ethanol withdrawal. Pharmacol. Biochem. Behav. *102*: 44–47.

Valentino, R.J., and Bockstaele, E. Van (2015). Endogenous Opioids: The Downside of Opposing Stress. Neurobiol. Stress *1*: 23–32.

Vanderah, T.W., Suenaga, N.M., Ossipov, M.H., Malan, T.P.J., Lai, J., and Porreca, F. (2001). Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. J. Neurosci. *21*: 279–286.

Vanegas, H., and Schaible, H.G. (2004). Descending control of persistent pain: Inhibitory or facilitatory? Brain Res. Rev. *46*: 295–309.

Vaughan, C.W., Ingram, S.L., Connor, M.A., and Christie, M.J. (1997). How opioids inhibit GABA-mediated neurotransmission. Nature *390*: 611–614.

Velly, A.M., and Mohit, S. (2018). Epidemiology of pain and relation to psychiatric disorders. Prog. Neuro-Psychopharmacology Biol. Psychiatry *87*: 159–167.

Vera-Portocarrero, L.P., Zhang, E.T., King, T., Ossipov, M.H., Vanderah, T.W., Lai, J., et al. (2007). Spinal NK-1 receptor expressing neurons mediate opioid-induced hyperalgesia and antinociceptive tolerance via activation of descending pathways. Pain *129*: 35–45.

Vergura, R., Balboni, G., Spagnolo, B., Gavioli, E., Lambert, D.G., McDonald, J., et al. (2008). Anxiolytic- and antidepressant-like activities of H-Dmt-Tic-NH-CH(CH2-COOH)-Bid (UFP-512), a novel selective delta opioid receptor agonist. Peptides *29*: 93–103.

Vunck, S.A., Snider, S.E., Oord, E.J.C.G. van den, and Beardsley, P.M. (2011). The kappa opioid receptor agonist, U50,488, exacerbates conditioned fear in mice. FASEB J. *25*: 617-620.

Waksman, G., Hamel, E., Fournie-Zaluski, M.C., and Roques, B.P. (1986). Autoradiographic comparison of the distribution of the neutral endopeptidase ‘enkephalinase’ and of mu and delta opioid receptors in rat brain. Proc. Natl. Acad. Sci. U. S. A. *83*: 1523–1527.

Walf, A.A., and Frye, C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. *2*: 322–328.

Walker, D.J., Zacny, J.P., Galva, K.E., and Lichtor, J.L. (2001). Subjective, psychomotor, and physiological effects of cumulative doses of mixed-action opioids in healthy volunteers. Psychopharmacology (Berl). *155*: 362–371.

Walker, J.R., Su, A.I., Self, D.W., Hogenesch, J.B., Lapp, H., Maier, R., et al. (2004). Applications of a rat multiple tissue gene expression data set. Genome Res. *14*: 742–749.

Walker, L.S., Sherman, A.L., Bruehl, S., Garber, J., and Smith, C.A. (2012). Functional abdominal pain patient subtypes in childhood predict functional gastrointestinal disorders with chronic pain and psychiatric comorbidities in adolescence and adulthood. Pain *153*: 1798–1806.

Walsh, S.L., Strain, E.C., Abreu, M.E., and Bigelow, G.E. (2001). Enadoline, a selective kappa opioid agonist: comparison with butorphanol and hydromorphone in humans. Psychopharmacology (Berl). *157*: 151–162.

Wang, D. sheng, Tian, Z., Guo, Y. yan, Guo, H. liang, Kang, W. bo, Li, S., et al. (2015a). Anxiolytic-like effects of translocator protein (TSPO) ligand ZBD-2 in an animal model of chronic pain. Mol. Pain *11*: 1–10.

Wang, G.-Q., Cen, C., Li, C., Cao, S., Wang, N., Zhou, Z., et al. (2015b). Deactivation of

excitatory neurons in the prelimbic cortex via Cdk5 promotes pain sensation and anxiety. Nat. Commun. *6*: 7660.

Wang, H., and Wessendorf, M.W. (2002). μ- and δ-opioid receptor mRNAs are expressed in periaqueductal gray neurons projecting to the rostral ventromedial medulla. Neuroscience *109*: 619–634.

Wang, J., Goffer, Y., Xu, D., Tukey, D.S., Shamir, D.B., Eberle, S.E., et al. (2011). A single subanesthetic dose of ketamine relieves depression-like behaviors induced by neuropathic pain in rats. Anesthesiology *115*: 812–821.

Wang, J.B., Johnson, P.S., Imai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M., et al. (1994a). cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Lett. *348*: 75–79.

Wang, J.B., Johnson, P.S., Persico, A.M., Hawkins, A.L., Griffin, C.A., and Uhl, G.R. (1994b). Human mu opiate receptor. cDNA and genomic clones, pharmacologic characterization and chromosomal assignment. FEBS Lett. *338*: 217–222.

Wang, P.K., Cao, J., Wang, H., Liang, L., Zhang, J., Lutz, B.M., et al. (2015c). Short-Term Sleep Disturbance-Induced Stress Does not Affect Basal Pain Perception, but Does Delay Postsurgical Pain Recovery. J. Pain *16*: 1186–1199.

Wang, R., King, T., Felice, M. De, Guo, W., Ossipov, M.H., and Porreca, F. (2013a). Descending facilitation maintains long-term spontaneous neuropathic pain. J. Pain *14*: 845–853.

Wang, S.-C., Chen, Y.-C., Lee, C.-H., and Cheng, C.-M. (2019). Opioid Addiction, Genetic Susceptibility, and Medical Treatments: A Review. Int. J. Mol. Sci. *20*:.

Wang, S., Tian, Y., Song, L., Lim, G., Tan, Y., You, Z., et al. (2012). Exacerbated mechanical hyperalgesia in rats with genetically predisposed depressive behavior: role of melatonin and NMDA receptors. Pain *153*: 2448–2457.

Wang, W., Qi, W.J., Xu, Y., Wang, J.Y., and Luo, F. (2010). The differential effects of depression on evoked and spontaneous pain behaviors in olfactory bulbectomized rats. Neurosci. Lett. *472*: 143–147.

Wang, X.-Q., Zhong, X.-L., Li, Z.-B., Wang, H.-T., Zhang, J., Li, F., et al. (2015d). Differential roles of hippocampal glutamatergic receptors in neuropathic anxiety-like behavior after partial sciatic nerve ligation in rats. BMC Neurosci. *16*: 14.

Wang, Y.J., Hang, A., Lu, Y.C., Long, Y., Zan, G.Y., Li, X.P., et al. (2016). κ Opioid receptor activation in different brain regions differentially modulates anxiety-related behaviors in mice. Neuropharmacology *110*: 92–101.

Wang, Z., Gardell, L.R., Ossipov, M.H., Vanderah, T.W., Brennan, M.B., Hochgeschwender, U., et al. (2001). Pronociceptive Actions of Dynorphin Maintain Chronic Neuropathic Pain. J. Neurosci. *21*: 1779–1786.

Wang, Z., Ocampo, M.A., Pang, R.D., Bota, M., Bradesi, S., Mayer, E.A., et al. (2013b). Alterations in prefrontal-limbic functional activation and connectivity in chronic stress-induced visceral hyperalgesia. PLoS One *8*: e59138.

Watkins, L.R., Wiertelak, E.P., Goehler, L.E., Mooney-Heiberger, K., Martinez, J., Furness, L., et al. (1994). Neurocircuitry of illness-induced hyperalgesia. Brain Res. *639*: 283–299.

Watson, G.S., Sufka, K.J., and Coderre, T.J. (1997). Optimal scoring strategies and

weights for the formalin test in rats. Pain *70*: 53–58.

Wei, X., Sun, Y., and Luo, F. (2017). Impaired Spinal Glucocorticoid Receptor Signaling Contributes to the Attenuating Effect of Depression on Mechanical Allodynia and Thermal Hyperalgesia in Rats with Neuropathic Pain. Front. Cell. Neurosci. *11*: 145.

Whitlock, E.L., Diaz-Ramirez, L.G., Glymour, M.M., Boscardin, W.J., Covinsky, K.E., and Smith, A.K. (2017). Association Between Persistent Pain and Memory Decline and Dementia in a Longitudinal Cohort of Elders. JAMA Intern. Med. *177*: 1146–1153.

Wiech, K., and Tracey, I. (2009). The influence of negative emotions on pain: behavioral effects and neural mechanisms. Neuroimage *47*: 987–994.

Will, C.C., Aird, F., and Redei, E.E. (2003). Selectively bred Wistar-Kyoto rats: an animal model of depression and hyper-responsiveness to antidepressants. Mol. Psychiatry *8*: 925–932.

Williams, J.T., Egan, T.M., and North, R.A. (1982). Enkephalin opens potassium channels on mammalian central neurones. Nature *299*: 74–77.

Willis, W.D., and Westlund, K.N. (1997). Neuroanatomy of the pain system and of the pathways that modulate pain. J. Clin. Neurophysiol. *14*: 2–31.

Willis, W.D.J. (1985). Pain pathways in the primate. Prog. Clin. Biol. Res. *176*: 117–133.

Willis, W.D.J. (1988). Anatomy and physiology of descending control of nociceptive responses of dorsal horn neurons: comprehensive review. Prog. Brain Res. *77*: 1–29.

Willner, P., Gruca, P., Lason, M., Tota-Glowczyk, K., Litwa, E., Niemczyk, M., et al. (2019). Validation of chronic mild stress in the Wistar-Kyoto rat as an animal model of treatment-resistant depression. Behav. Pharmacol. *30*: 239–250.

Winston, J.H., Li, Q., and Sarna, S.K. (2014). Chronic prenatal stress epigenetically modifies spinal cord BDNF expression to induce sex-specific visceral hypersensitivity in offspring. Neurogastroenterol. Motil. *26*: 715–730.

Wittmann, W., Schunk, E., Rosskothen, I., Gaburro, S., Singewald, N., Herzog, H., et al. (2009). Prodynorphin-derived peptides are critical modulators of anxiety and regulate neurochemistry and corticosterone. Neuropsychopharmacology *34*: 775–785.

Woolf, C.J. (1983). Evidence for a central component of post-injury pain hypersensitivity. Nature *306*: 686–688.

Woolf, C.J., and Ma, Q. (2007). Nociceptors--noxious stimulus detectors. Neuron *55*: 353–364.

Wouters, M.M., Wanrooy, S. Van, Casteels, C., Nemethova, A., Vries, A. de, Oudenhove, L. Van, et al. (2012). Altered brain activation to colorectal distention in visceral hypersensitive maternal-separated rats. Neurogastroenterol. Motil. *24*: 678–85, e297.

Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G.W., Vardy, E., et al. (2012). Structure of the human kappa-opioid receptor in complex with JDTic. Nature *485*: 327–332.

Wu, Y.Y., Yao, X.M., Jiang, Y.L., He, X.F., Shao, X.M., Du, J.Y., et al. (2017). Pain aversion and anxiety-like behavior occur at different times during the course of chronic inflammatory pain in rats. J. Pain Res. *10*: 2585–2593.

Wyss, J.M., Chambless, B.D., Kadish, I., and Groen, T. Van (2000). Age-related decline in water maze learning and memory in rats: Strain differences. Neurobiol. Aging *21*: 671–

681.

Xie, J.Y., Felice, M. De, Kopruszinski, C.M., Eyde, N., LaVigne, J., Remeniuk, B., et al. (2017). Kappa opioid receptor antagonists: A possible new class of therapeutics for migraine prevention. Cephalalgia *37*: 780–794.

Yaksh, T.L., Yeung, J.C., and Rudy, T.A. (1976a). Systematic examination in the rat of brain sites sensitive to the direct application of morphine: observation of differential effects within the periaqueductal gray. Brain Res. *114*: 83–103.

Yaksh, T.L., Yeung, J.C., and Rudy, T.A. (1976b). Systematic examination in the rat of brain sites sensitive to the direct application of morphine: Observation of differential effects within the periaqueductal gray. Brain Res. *114*: 83–103.

Yalcin, I., Aksu, F., and Belzung, C. (2005). Effects of desipramine and tramadol in a chronic mild stress model in mice are altered by yohimbine but not by pindolol. Eur. J. Pharmacol. *514*: 165–174.

Yalcin, I., Belzung, C., and Surget, A. (2008). Mouse strain differences in the unpredictable chronic mild stress: a four-antidepressant survey. Behav. Brain Res. *193*: 140–143.

Yalcin, I., Bohren, Y., Waltisperger, E., Sage-Ciocca, D., Yin, J.C., Freund-Mercier, M.J., et al. (2011). A time-dependent history of mood disorders in a murine model of neuropathic pain. Biol. Psychiatry *70*: 946–953.

Yamada, K., and Nabeshima, T. (1995). Stress-induced behavioral responses and multiple opioid systems in the brain. Behav. Brain Res. *67*: 133–145.

Yamazaki, Y., Maeda, T., Someya, G., and Wakisaka, S. (2001). Temporal and spatial distribution of Fos protein in the lumbar spinal dorsal horn neurons in the rat with chronic constriction injury to the sciatic nerve. Brain Res. *914*: 106–114.

Yang, L., Xin, X., Zhang, J., Zhang, L., Dong, Y., Zhang, Y., et al. (2014). Inflammatory pain may induce cognitive impairment through an interlukin-6-dependent and postsynaptic density-95-associated mechanism. Anesth. Analg. *119*: 471–480.

Yang, Q.Z., Lu, S.S., Tian, X.Z., Yang, A.M., Ge, W.W., and Chen, Q. (2011). The antidepressant-like effect of human opiorphin via opioid-dependent pathways in mice. Neurosci. Lett. *489*: 131–135.

Yasuda, K., Espinosa, R., Takeda, J., Beau, M.M. Le, and Bell, G.I. (1994). Localization of the kappa opioid receptor gene to human chromosome band 8q11.2. Genomics *19*: 596–597.

Yeung, J.C., Yaksh, T.L., and Rudy, T.A. (1977). Concurrent mapping of brain sites for sensitivity to the direct application of morphine and focal electrical stimulation in the production of antinociception in the rat. Pain *4*: 23–40.

Yoshida, A., Chen, K., Moritani, M., Yabuta, N.H., Nagase, Y., Takemura, M., et al. (1997). Organization of the descending projections from the parabrachial nucleus to the trigeminal sensory nuclear complex and spinal dorsal horn in the rat. J. Comp. Neurol. *383*: 94–111.

Yoshino, A., Okamoto, Y., Onoda, K., Shishida, K., Yoshimura, S., Kunisato, Y., et al. (2012). Sadness enhances the experience of pain and affects pain-evoked cortical activities: An MEG study. J. Pain *13*: 628–635.

Yoshino, A., Okamoto, Y., Onoda, K., Yoshimura, S., Kunisato, Y., Demoto, Y., et al.

(2010). Sadness enhances the experience of pain via neural activation in the anterior cingulate cortex and amygdala: An fMRI study. Neuroimage *50*: 1194–1201.

Young, E.G., Watkins, L.R., and Mayer, D.J. (1984). Comparison of the effects of ventral medullary lesions on systemic and microinjection morphine analgesia. Brain Res. *290*: 119–129.

Yue, J., Wang, X. shang, Guo, Y. yan, Zheng, K. yin, Liu, H. yan, Hu, L. ning, et al. (2018). Anxiolytic effect of CPEB1 knockdown on the amygdala of a mouse model of inflammatory pain. Brain Res. Bull. *137*: 156–165.

Zachariou, V., and Goldstein, B.D. (1996a). Kappa-opioid receptor modulation of the release of substance P in the dorsal horn. Brain Res. *706*: 80–88.

Zachariou, V., and Goldstein, B.D. (1996b). δ-Opioid receptor modulation of the release of substance P-like immunoreactivity in the dorsal horn of the rat following mechanical or thermal noxious stimulation. Brain Res. *736*: 305–314.

Zadina, J.E., Hackler, L., Ge, L.J., and Kastin, A.J. (1997). A potent and selective endogenous agonist for the mu-opiate receptor. Nature *386*: 499–502.

Zafar, H.M., Pare, W.P., and Tejani-Butt, S.M. (1997). Effect of acute or repeated stress on behavior and brain norepinephrine system in Wistar-Kyoto (WKY) rats. Brain Res. Bull. *44*: 289–295.

Zarrindast, M.-R., Rostami, P., Zarei, M., and Roohbakhsh, A. (2005). Intracerebroventricular effects of histaminergic agents on morphine-induced anxiolysis in the elevated plus-maze in rats. Basic Clin. Pharmacol. Toxicol. *97*: 276–281.

Zarrindast, M.R., and Zarghi, A. (1992). Morphine stimulates locomotor activity by an indirect dopaminergic mechanism: Possible D-1 and D-2 receptor involvement. Gen. Pharmacol. *23*: 1221–1225.

Zastawny, R.L., George, S.R., Nguyen, T., Cheng, R., Tsatsos, J., Briones-Urbina, R., et al. (1994). Cloning, characterization, and distribution of a mu-opioid receptor in rat brain. J. Neurochem. *62*: 2099–2105.

Zautra, A.J., Parrish, B.P., Puymbroeck, C.M. Van, Tennen, H., Davis, M.C., Reich, J.W., et al. (2007). Depression history, stress, and pain in rheumatoid arthritis patients. J. Behav. Med. *30*: 187–197.

Zeng, Q., Wang, S., Lim, G., Yang, L., Mao, J., Sung, B., et al. (2008). Exacerbated mechanical allodynia in rats with depression-like behavior. Brain Res. *1200*: 27–38.

Zhang-James, Y., Middleton, F.A., and Faraone, S. V (2013). Genetic architecture of Wistar-Kyoto rat and spontaneously hypertensive rat substrains from different sources. Physiol. Genomics *45*: 528–538.

Zhang-James, Y., Yang, L., Middleton, F.A., Yang, L., Patak, J., and Faraone, S. V. (2014). Autism-related behavioral phenotypes in an inbred rat substrain. Behav. Brain Res. *269*: 103–114.

Zhang, D.X., Carlton, S.M., Sorkin, L.S., and Willis, W.D. (1990). Collaterals of primate spinothalamic tract neurons to the periaqueductal gray. J. Comp. Neurol. *296*: 277–290.

Zhang, G.F., Wang, J., Han, J.F., Guo, J., Xie, Z.M., Pan, W., et al. (2016). Acute single dose of ketamine relieves mechanical allodynia and consequent depression-like behaviors in a rat model. Neurosci. Lett. *631*: 7–12.

Zhang, H., Shi, Y.G., Woods, J.H., Watson, S.J., and Ko, M.C. (2007). Central κ-opioid receptor-mediated antidepressant-like effects of nor-Binaltorphimine: Behavioral and BDNF mRNA expression studies. Eur. J. Pharmacol. *570*: 89–96.

Zhang, L., Zhang, Y., and Zhao, Z.-Q. (2005a). Anterior cingulate cortex contributes to the descending facilitatory modulation of pain via dorsal reticular nucleus. Eur. J. Neurosci. *22*: 1141–1148.

Zhang, R.-X., and Ren, K. (2011). Animal Models of Inflammatory Pain. In Animal Models of Pain, C. Ma, and J.-M. Zhang, eds. (New York: Humana Press), pp 23–40.

Zhang, Y., Butelman, E.R., Schlussman, S.D., Ho, A., and Kreek, M.J. (2005b). Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: agonist actions at kappa opioid receptors. Psychopharmacology (Berl). *179*: 551–558.

Zhang, Y., Gandhi, P.R., and Standifer, K.M. (2012). Increased nociceptive sensitivity and nociceptin/orphanin FQ levels in a rat model of PTSD. Mol. Pain *8*: 1.

Zhang, Y., Meng, X., Li, A., Xin, J., Berman, B.M., Lao, L., et al. (2011). Acupuncture alleviates the affective dimension of pain in a rat model of inflammatory hyperalgesia. Neurochem. Res. *36*: 2104–2110.

Zhang, Y., Simpson-Durand, C.D., and Standifer, K.M. (2015). Nociceptin/orphanin FQ peptide receptor antagonist JTC-801 reverses pain and anxiety symptoms in a rat model of post-traumatic stress disorder. Br. J. Pharmacol. *172*: 571–582.

Zhang, Y.Q., Gao, X., Zhang, L.M., and Wu, G.C. (2000). The release of serotonin in rat spinal dorsal horn and periaqueductal gray following carrageenan inflammation. Neuroreport *11*: 3539–3543.

Zhang, Z., Xin, S.M., Wu, G.X., Zhang, W.B., Ma, L., and Pei, G. (1999). Endogenous delta-opioid and ORL1 receptors couple to phosphorylation and activation of p38 MAPK in NG108-15 cells and this is regulated by protein kinase A and protein kinase C. J. Neurochem. *73*: 1502–1509.

Zhao, J., Gao, X., Wang, A., Wang, Y., Du, Y., Li, L., et al. (2019). Depression comorbid with hyperalgesia: Different roles of neuroinflammation induced by chronic stress and hypercortisolism. J. Affect. Disord. *256*: 117–124.

Zhao, X., Wang, C., Zhang, J.-F., Liu, L., Liu, A.-M., Ma, Q., et al. (2014). Chronic curcumin treatment normalizes depression-like behaviors in mice with mononeuropathy: involvement of supraspinal serotonergic system and GABAA receptor. Psychopharmacology (Berl). *231*: 2171–2187.

Zhao, Y.-L., Chen, S.-R., Chen, H., and Pan, H.-L. (2012). Chronic opioid potentiates presynaptic but impairs postsynaptic N-methyl-D-aspartic acid receptor activity in spinal cords: implications for opioid hyperalgesia and tolerance. J. Biol. Chem. *287*: 25073–25085.

Zhong, F., Liu, L., Wei, J.L., Hu, Z.L., Li, L., Wang, S., et al. (2019). Brain-derived neurotrophic factor precursor in the hippocampus regulates both depressive and anxiety-like behaviors in rats. Front. Psychiatry *10*: 1–11.

Zhu, C. Bin, Cao, X.D., Xu, S.F., and Wu, G.C. (1997). Orphanin FQ potentiates formalin-induced pain behavior and antagonizes morphine analgesia in rats. Neurosci. Lett. *235*: 37–40.

Zhuo, M. (2016). Neural Mechanisms Underlying Anxiety-Chronic Pain Interactions. Trends Neurosci. *39*: 136–145.

Zhuo, M. (2017). Descending facilitation. Mol. Pain *13*: 1744806917699212.

Zomkowski, A.D.E., Santos, A.R.S., and Rodrigues, A.L.S. (2005). Evidence for the involvement of the opioid system in the agmatine antidepressant-like effect in the forced swimming test. Neurosci. Lett. *381*: 279–283.

Zubieta, J., Smith, Y., Bueller, J., Xu, Y., Kilbourn, M., Jewett, D., et al. (2001). Regional mu opioid receptor regulation of sensory and affective dimensions of pain. Science *293*: 311–5.

Zyl, P.J. van, Dimatelis, J.J., and Russell, V.A. (2014). Changes in behavior and ultrasonic vocalizations during antidepressant treatment in the maternally separated Wistar-Kyoto rat model of depression. Metab. Brain Dis. *29*: 495–507.