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Title	Regulation of fear, anxiety and cognition in the presence or absence of pain by peroxisome proliferator-activated receptors
Author(s)	Gaspar, Jessica
Publication Date	2019-06-07
Publisher	NUI Galway
Item record	http://hdl.handle.net/10379/15218

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Regulation of fear, anxiety and cognition in the presence or absence of pain by peroxisome proliferator-activated receptors

Jessica Cristina Coelho Gaspar, B.Sc.

Pharmacology and Therapeutics, School of Medicine

NCBES Galway Neuroscience 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

Dr. Michelle Roche

Physiology, School of Medicine, National University or Ireland, Galway

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

April 2019

Contents

Abstract

Fear/anxiety and pain modulate one another reciprocally, but the neurobiological mechanisms that underlie this interaction are not completely understood. Fear-conditioned analgesia (FCA) is pain suppression upon exposure to a fearful stimulus. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that modulate in pain, anxiety, and cognition. However, their role in pain-fear/anxiety interactions is unknown. The basolateral amygdala (BLA) and the central nucleus of the amygdala (CeA) play a key role in pain, conditioned fear and FCA. The overall aim of this thesis was to investigate the role of PPARα, PPARβ/δ and PPARγ in acute and chronic inflammatory pain, conditioned fear, FCA, anxiety and cognition. In addition, the influence of pain on PPAR-mediated modulation of conditioned fear, innate anxiety and cognition was investigated.

The FCA protocol combined footshocks with context and formalin-injection into the hind paw. On conditioning days, Male Sprague-Dawley rats received footshocks in a conditioning arena, while control rats were placed in the arena for an equivalent amount of time (9min 30secs; no footshocks). 23.5 hours later, rats received an intraplantar injection of formalin into the right hind paw. Rats received either intraperitoneal or intra-amygdalar injection of vehicle or PPARα, PPARβ/δ or PPARγ antagonists prior to re-exposure to the arena and pain and fear-related behaviours were recorded for 15 or 30 minutes. In the final study, rats received intraplantar injections of complete Freund's adjuvant (CFA), or only needle insertion, into the right hind paw; they received an intraperitoneal injection of vehicle, PEA, or PPARα, PPARβ/δ or PPARγ antagonists and underwent pain (von Frey) and anxiety (elevated plus maze, open field, and light-dark box) tests on days 1, 7, 21 and 28 (pain) and 21 (anxiety), and a novel object recognition protocol on days 26-28.

The key results indicated that the blockade of PPAR signalling, particularly PPARα and PPARγ in the BLA, but not CeA, prolonged or enhanced contextually induced freezing behaviour in the presence of formalin-evoked nociceptive tone. In the absence of nociceptive tone, the blockade of PPARs in the BLA increased freezing expression in non-fear-conditioned rats, indicating a possible modulatory role of PPARs in innate anxiety. These results were associated with increased tissue levels of dopamine in the right BLA. The systemic administration of a PPARα antagonist impaired spatial memory of rats in the presence, but not in the absence, of chronic inflammatory pain induced by CFA. Systemic, intra-BLA or intra-CeA administration of PPAR antagonists did not alter formalin-evoked

nociceptive behaviour, FCA or mechanical allodynia in the CFA model.

In conclusion, these findings indicate a key role for PPARs in the BLA in mediating and modulating innate and conditioned fear behaviour, effects dependant on the presence or absence of nociceptive stimuli. Furthermore, PPARα signalling appears to enhance deficits in cognitive responses in the presence of chronic inflammatory pain. Taken together these data add to the body of knowledge on the role of PPARs in pain, fear and cognition and their interactions.

Author's declaration

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

Chapter 2

- Assisted by Dr. Bright Okine during the behavioural tests.
- The mass spectrometry of the ventral hippocampus was part of Laura Boullon's Summer project
- Assisted by Brendan Harhen for mass spectrometry procedure and analysis

Chapter 3

- The behavioural analysis was done by David Dineen as part of his 4th year BSc project
- Laura Boullon assisted with generation of the western blotting images of PPARα
- Assisted by Dr. Bright Okine during the surgeries

Chapter 5

- Assisted by Mehnaz Ferdousi 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:_____

Date:_____

Acknowledgements

Firstly, I would like to thank my supervisor Professor David Finn for the support, patience, encouragement, and mentorship during the years. Also, thank you for the opportunity you gave me when you accepted me in your research group, because these years certainly changed my life. I would also like to thank my co-supervisor Dr. Michelle Roche for her guidance and support during these years.

A special thank you to CNPq – Brazil for the financial support through the program Science without Borders.

To everyone in CNS, you all have been the best group of people I have worked with. It was not only a pleasure, but a privilege to work, laugh, drink, party and complain with you all! There was never a time when I did not have the support of one of you when needed. Thank you all so much!

To the Finns: what a journey, guys! To all and each of you, my most sincere thank you and my wishes of a very successful professional and personal life wherever you go. I could not have found a more supportive group. None of you will ever be forgotten!

Dr. Daniel Kerr, thank you so much! You were a fantastic teacher and a great advisor! My favourite Irish (do not tell that to Patrick Harper)!

Dr. Bright Okine, no words to say how thankful I am! You were my mentor and my guide, thank you so much for everything!

To Ambrose, GRC members and all the staff of the Pharmacology Department my thank you for all the support over the years.

Diego, esta conquista é tão tua quanto minha. Eu não teria chego tão longe sem você na minha vida. Eu não tenho palavras pra agradecer o amor, o carinho, a confiança e o apoio que tu me destes nestes anos. Só espero que eu consiga retribuir um pouquinho ao longo dos anos. Eu não poderia ter escolhido alguém melhor para passar o resto da minha vida. Eu te amo!

Rafa, Mamãe e Helton: vocês sempre foram os meus maiores apoiadores em tudo, desde sempre. Obrigado por terem me apoiado em mais esse desafio e por terem aguentado comigo esta tortura que foi ficarmos tão longe. Eu não tenho palavras pra descrever o tamanho do meu amor por vocês, e o quanto são importantes na minha vida! Amo vocês!

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List of abbreviations

2-AG – 2-Arachidonoylglycerol

5-HT – Serotonin

ACC – Anterior cingulate cortex

AD – Alzheimer's disease

AEA – Anandamide

BLA – Basolateral amygdala

BMA – Basomedial amygdala

BNST – Bed nucleus of the stria terminalis

CA – *Cornu ammonis*

CB_1 – Cannabinoid receptor 1

CBT - Cognitive behavioural therapy

CCI – Chronic constriction injury

CeA – Central nucleus of the amygdala

CEC - Capsular subdivision of the central amygdala

CFA – Complete Freund's adjuvant

CFC – Context fear conditioning

CNS – Central nervous system

COX-2 - cyclooxygenase-2

CPS – Composite pain score

CS – Conditioned stimulus

D_2 – Dopaminergic receptor 2

DA – Dopamine

DG – Dentate Gyrus

DH – Dorsal Hippocampus

dlPAG – Dorsolateral periaqueductal grey

dmPAG – Dorsomedial periaqueductal grey

dmPFC – Dorsomedial prefrontal cortex

DMSO - Dimethyl sulfoxide

DRG – Dorsal Root Ganglia

EMSA - electrophoretic mobility-shift assay

EPM – Elevated Plus Maze

EPSC(s) - Excitatory postsynaptic current(s)

FAAH - fatty acid amide hydrolase

FC – Fear-conditioned

FCA – Fear-conditioned analgesia

GSD - Generalised stress disorder

HPLC - High pressure liquid chromatography

i.p. – Intraperitoneal

IASP - International Association for the Study of Pain

IF – Infralimbic cortex

IHC – Immunohistochemistry

iNOS - nitric oxide synthase

ITC – Intercalated cells of the amygdala

LA – Lateral amygdala

lCeA – Lateral central amygdala

LC-MS/MS - Liquid chromatography coupled to tandem mass spectrometry

LDB – Light-Dark Box

LHyp – Lateral hypothalamus

lPAG – Lateral periaqueductal grey

LPS – Lipopolysaccharide

mCeA – Medial central amygdala

mPFC – Medial prefrontal cortex

MS – Multiple Sclerosis

NAE(s) - N-acylethanolamine(s)

NFC – Non-fear conditioned

NF-$_{K}$B - Nuclear Factor kappa-light-chain-enhancer of activated B cells

NOR – Novel Object Recognition

NSAID(s) - Non-steroidal anti-inflammatory drug(s)

OCD - Obsessive-compulsive disorder

OEA – and N-oleoylethanolamide

OF – Open Field

PAG – Periaqueductal Grey

PEA – N-palmitoylethanolamide

PEA-μm - micronized PEA

PFC – Prefrontal Cortex

PL – Prelimbic cortex

PN – Parabrachial nucleus

PPAR(s) – Peroxisome proliferator-activated receptor(s)

PPARα - Peroxisome proliferator-activated receptor α

PPARβ/δ - Peroxisome proliferator-activated receptor β/δ

PPARγ - Peroxisome proliferator-activated receptor γ

PPRE - Peroxisome proliferator response element

PTSD – Posttraumatic stress disorder

PVN – Paraventricular nucleus of the thalamus

QC – Quality Control

RT-qPCR - Quantitative real-time polymerase chain reaction

RVM - Rostral ventromedial medulla

RXR - retinoid X receptor

S.E.M. – Standard error of the mean

SD – Sprague-Dawley

SDev – Standard Deviation

SIA – Stress-induced analgesia

SNI – Spared nerve injury

SNRI(s) - Serotonin-noradrenaline reuptake inhibitor(s)

SSRI(s) - selective serotonin reuptake inhibitor(s)

TH – Thalamus

TNF-α - tumour necrosis factor- α

$TRPV_1$ – Transient receptor potential cation channel subfamily V member 1

US – Unconditioned stimulus

VH – Ventral Hippocampus

vlPAG – Ventrolateral periaqueductal grey

VTA – Ventral Tegmental Area

WB – Western Blotting

WKY – Wistar-Kyoto

WT – Wild Type

List of publications and Conferences proceedings

Publications - Peer-reviewed journal articles

- **Reviews**

1. Okine BN*, **Gaspar JC***, Finn DP (2018) PPARs and pain. British Journal of Pharmacology.

*Shared first authorship

- **Original Research articles**

1. Okine BN, **Gaspar JC**, Madasu MK, Olango WM, Harhen B, Roche M, Finn DP (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 Research 15:185-192.

2. Okine BN, Madasu MK, McGowan F, Prendergast C, **Gaspar JC**, Harhen B, Roche M, Finn DP (2016) N-palmitoylethanolamide in the anterior cingulate cortex attenuates inflammatory pain behaviour indirectly via a CB1 receptor-mediated mechanism. Pain 157(12) 2687 – 2696.

Original Research articles (submitted)

1. **Gaspar JC**, Okine BN, Llorente-Berzal A, Roche M, Finn DP. Pharmacological blockade of PPAR isoforms increases conditioned fear responding in the presence of nociceptive tone. [submitted]
2. Okine BN, McLaughlin G, **Gaspar JC**, Harhen B, Roche M, Finn DP. GPR55 receptor signalling in the anterior cingulate cortex facilitates inflammatory pain. [submitted]

Original Research articles (in preparation)

1. **Gaspar JC**, Okine BN, Dinneen D, Roche M, Finn DP (2019) Intra-BLA administration of PPAR antagonists has differential effects on conditioned fear in the presence or absence of nociceptive tone in rats.

2. **Gaspar JC**, Roche M, Finn DP (2019) Systemic administration of PPARα antagonist GW6471 affects spatial memory in the presence of chronic inflammatory pain.
3. Llorente-Berzal A, McGowan F, **Gaspar JC**, Rea K, Roche M, Finn DP (2019) Sexually dimorphic expression of fear-conditioned analgesia in rats and associated alterations in the endocannabinoid system in the periaqueductal grey

Conference Proceedings/Abstracts

Refereed or published conference abstracts/proceedings

1. **Gaspar JC**, Okine BN, Llorente-Berzal A, Dinneen D, Roche M, Finn DP (2019) Differential role of amygdala PPARs in conditioned fear-related behaviour in the presence or absence of nociceptive tone. British Neuroscience Association. Dublin, Ireland, April 2019 Poster presentation

2. **Gaspar JC**, Okine BN, Llorente-Berzal A, Dinneen D, Roche M, Finn DP (2018) Differential role of amygdala PPARs in conditioned fear-related behaviour in the presence or absence of nociceptive tone. *Society of Neuroscience Scientific Meeting. San Diego, USA.* November 2018 Poster presentation

3. **Gaspar JC**, Okine BN, Llorente-Berzal A, Dinneen D, Roche M, Finn DP (2018) The effects of pharmacological blockade of PPARs on formalin-evoked nociceptive behaviour, fear conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *11th FENS - Forum of Neuroscience Scientific Meeting*. July 2018 Poster presentation

4. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Dinneen D, Roche M, Finn DP (2017) The effects of pharmacological blockade of PPARs on formalin-evoked nociceptive behaviour, fear conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *27th Annual Symposium of the International Cannabinoid Research Society,* P2-10 Poster Presentation

5. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Dinneen D, Roche M, Finn DP (2017) The effects of pharmacological blockade of PPARs on formalin-evoked nociceptive behaviour, fear conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *8th European Workshop on Cannabinoid Research Society*, P45 Poster Presentation

6. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Dinneen D, Roche M, Finn DP (2017) The effects of pharmacological blockade of PPARs on formalin-evoked nociceptive behaviour, fear conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *10th Neuroscience Ireland Conference* Poster presentation
7. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Dinneen D, Roche M, Finn DP (2017) The effects of pharmacological blockade of PPARs on formalin-evoked

nociceptive behaviour, fear conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *Irish Pain Society Annual Scientific Meeting* Poster presentation

Non-Refereed/Non-Published Abstracts/Proceedings:

1. **Gaspar JC**, Okine BN, Llorente-Berzal A, Dinneen D, Roche M, Finn DP (2019) Differential role of amygdala PPARs in conditioned fear-related behaviour in the presence or absence of nociceptive tone *Centre of Pain Research Annual Research day, Galway Ireland*. March 2019

2. **Gaspar JC**, Okine BN, Llorente-Berzal A, Dinneen D, Roche M, Finn DP (2018) The effects of pharmacological blockade of PPARs on formalin-evoked nociceptive behaviour, fear conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *Young Neuroscience Ireland Symposium, Dublin Ireland.* October 2018 Poster presentation

3. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Dinneen D, Roche M, Finn DP (2018) The effects of pharmacological blockade of PPARs on formalin-evoked nociceptive behaviour, fear conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *College of Medicine, Nursing and Health Sciences postgraduate Research Day, Galway Ireland*. May 2016 Poster presentation

4. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Roche M, Finn DP (2016). Investigating the effects of pharmacological blockade of PPARα and PPARβ/δ on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *Centre of Pain Research annual research day, Galway Ireland*. March 2016. Poster

5. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Roche M, Finn DP (2016). Investigating the effects of pharmacological blockade of PPARα and PPARβ/δ on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *College of Medicine, Nursing and Health Sciences postgraduate Research Day, Galway Ireland.* June 2016. Poster

6. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Roche M, Finn DP (2016). Investigating the effects of pharmacological blockade of PPARα and PPARβ/δ on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *Encontro da associação deos Estudantes Brasileiros no Reino Unido ABEP-UK, London UK May 2016*. Poster

7. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Roche M, Finn DP (2016). Investigating the effects of pharmacological blockade of PPARα and PPARβ/δ on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *Neuroscience Ireland Young Neuroscientists Symposium, Dublin Ireland. September 2016*. Poster

8. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Roche M, Finn DP (2016). Investigating the effects of pharmacological blockade of PPARα and PPARβ/δ on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *Irish Pain Society Meeting, Dublin Ireland. September 2016*. Poster
9. Okine BN, **Gaspar JC**, Madasu MK, Olango WM, Harhen B, Roche M, Finn DP (2016) Characterisation of peroxisome proliferator-activated receptor signalling in the midbrain periaqueductal grey of rats genetically prone to heightened stress, negative affect and hyperalgesia. *Irish Pain Society Meeting, Dublin Ireland, September 2016*. Poster

10. Okine BN, **Gaspar JC**, Madasu MK, Olango WM, Harhen B, Roche M, Finn DP (2016) Characterisation of peroxisome proliferator-activated receptor signalling in the midbrain periaqueductal grey of rats genetically prone to heightened stress, negative affect and hyperalgesia. *Galway Neuroscience Centre's Research Day, Galway Ireland, December 2016*. Poster

11. **Gaspar JC**, Okine BN, Llorente-Berzal A, Burke O, Dineen D, Roche M, Finn DP (2016). The effects of pharmacological blockade of PPARs on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats. *Centre of Pain Research Annual Research day, Galway Ireland*. March 2017

1. Introduction

1.1 Anxiety and anxiety disorders

Anxiety refers to multiple psychological and physiological phenomena, including a conscious state of worry over a future unwanted event or fear of a situation (Evans et al., 2005). When mild, this mental state helps animal development, facilitating anticipation of certain situations in order to ensure safety and protection. However, when anxiety is excessive, it is maladaptive, with negative consequences for mental health. Anxiety disorders are the most prevalent mental illnesses in the European Union, with an estimated 36 to 60 million people being affected per year (Wittchen et al., 2011). According to the World Health Organisation in their document entitled *"Depression and other common mental disorders"* from 2017, 3.6% (264 million people) of the global population is affected by anxiety disorders. In Europe, 14.5% of the population are reported to have had experienced anxiety disorder-related episodes at least once in their lifetime, and around 10% are reported to have had one episode in the previous 12 months (Wittchen and Jacobi, 2005; Alonso et al., 2007; Bandelow and Michaelis, 2015). These anxiety disorder-related episodes include symptoms related to panic disorder, social anxiety disorder, separation anxiety disorder, obsessive-compulsive disorder (OCD), posttraumatic stress disorder (PTSD), generalised stress disorder (GSD), and specific phobias. Anxiety disorders are twice as likely to affect women and more prevalent in individuals under 35 years old (Bandelow and Michaelis, 2015; Remes et al., 2016). Anxiety disorders cost the European Union €41 billion in 2004 (Andlin-Sobocki and Wittchen, 2005). Additionally, it is projected that work loss due to anxiety is higher than for some somatic disorders, although it is important to point out that patients with anxiety disorders such as phobias and OCD do not often look for medical assistance, and prefer to hide their symptoms or avoid potential triggering situations (Bandelow and Michaelis, 2015).

Current treatments for anxiety include psychotherapies - the most widely used being cognitive behavioural therapy (CBT), but also relaxation, psychodynamic therapy, mindfulness meditation and others - and pharmacological therapy, including selective serotonin reuptake inhibitors (SSRIs), serotonin-noradrenaline reuptake inhibitors (SNRIs), tricyclic antidepressants, benzodiazepines, and others (Murrough et al., 2015). Studies have conflicting results when comparing these two types of intervention and it is not possible to affirm that one therapy is more effective than the other (see Bandelow et al., 2015 for a

review). The neurobiology and neurocircuitry of anxiety have been extensively investigated (see Section 1.3 below) and basic research has provided numerous insights into anxiety and fear behaviour and its underlying neurobiology. However, in the last two-to-three decades, few new mechanistic novel medications for anxiety disorders has been brought to market, and pharmacological treatments currently available have many side effects. For that reason, basic research aims to provide new insights into the neurobiology of anxiety and fear states and identify novel receptors and molecules that can be modulated for therapeutic benefit (see Murrough et al., 2015 for a review on new therapeutic targets for anxiety disorders).

1.2 Pain

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" by the International Association for the Study of Pain (IASP). Similar to anxiety, pain is important for the survival of organisms, serving to warn organisms of potential tissue damage. However, it can be debilitating when triggered or exacerbated in the absence of any noxious stimulus. Depending on its duration, pain can be classified as acute or chronic. Acute pain is of short duration while chronic pain in humans is defined as pain persisting for over 3 months. Several studies have estimated the economic and social cost associated with chronic pain in Europe (Breivik et al., 2006, 2013; Phillips, 2009; Gustavsson et al., 2012; Langley et al., 2013). The annual cost of treating chronic pain in Ireland was reported to be around €5.34 billion a year - €5,665 per patient (Raftery et al., 2012). Chronic pain afflicts almost one in five Europeans and is frequently associated with mood disorders. A study on the prevalence, impact and cost of chronic pain (PRIME) indicated a 35.5% prevalence of chronic pain in the Republic of Ireland (Raftery et al., 2011). Additionally, a few studies have shown figures of the prevalence of multimorbity (the occurrence of two or more chronic conditions at the same time) ranging from 27 to 66.2% in the Irish population (Slattery et al., 2017).

Current approaches to pain management include pharmacological therapies with opioid analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), local anaesthetics, anti-depressants, anti-convulsants and gabapentinoids, as well as non-pharmacological techniques such as acupuncture, meditation, physiotherapy and psychotherapy (Coutaux, 2017). The available pharmacotherapies for pain management are not always effective, and *circa* 40% of patients are unsatisfied with their treatment (Breivik et al., 2006). Many of the

above mentioned pharmacotherapies are associated with side effects such as constipation, nausea, vomiting, gastrointestinal irritation and ulceration, impaired cognitive abilities, loss of motor coordination, and anxiety (Khademi et al., 2016; Nakatani, 2017; Rayar et al., 2017). Furthermore, patients may develop tolerance or addiction to some of these drugs (Khademi et al., 2016; Nakatani, 2017).

Animals are often exposed to different noxious stimuli of varying intensity and quality. Specialised receptors (on nerve endings that innervate peripheral tissues) respond to different noxious stimuli, resulting in generation of action potentials and transmission of nociceptive information to the brain, which is involved in the interpretation of these signals and in the command of a proper response, when needed. The specialised neurons responsible for initiation of pain are called nociceptors (*nocere*, Latin for "hurt"), and have their cell bodies located in the dorsal root ganglia (DRG), and nerve terminals in the dorsal horn of the spinal cord. The peripheral endings of these nociceptors have a variety of specialised receptors which detect external stimuli. One important ion channel family that detects and transmits noxious stimuli is the transient receptor potential (TRP) channel family. The TRP family is divided into six subfamilies, classified as canonical (TRPC), vanilloid (TRPV), ankyrin (TRPA), melastatin (TRPM), polycystin (TRPP), and mucolipin (TRPML) (Wu et al., 2010). For instance, members of the TRPM subfamily detect noxious cold and members of the TRPV family detect noxious heat (Julius, 2013). Especially important for this thesis is the role of TRPA1, which is activated by formalin (McNamara et al., 2007). The activation of these receptors results in an influx of cations that ultimately results in an action potential. After being activated by a stimulus, the sensory information is sent through the primary afferent fibres to the dorsal horn of the spinal cord where they synapse with secondary sensory neurons in different laminae of the dorsal horn (Millan, 1999; Almeida et al., 2004). After decussation in the spinal cord, the nociceptive information is then relayed via these second order neurons to supraspinal regions via one of the ascending pathways (Willis, 1985; Almeida et al., 2004) (Figure 1.1).

The classification of nociceptors is based on the properties of their axons. The nociceptor with myelinated faster-conducting (rapid and sharp type of pain) axons are part of the Aδ–fibre group, and nociceptors with unmyelinated slower-conducting (slower, persisting pain) axons are part of the C-fibre group. After being activated by a stimulus, the sensorial information is sent through the primary afferent fibres to the dorsal horn of the spinal cord

where they make synapses with secondary sensory neurons in different laminae of the dorsal horn (Millan, 1999; Almeida et al., 2004). After decussation in the spinal cord, the nociceptive information is then relayed via these second order neurons to supraspinal regions via one of the ascending pathways (Willis, 1985; Almeida et al., 2004) (Figure 1.1).

1.2.1 *A*scending Pain Pathways

The bundles of ascending axons form two distinct phylogenetic systems. The older pathway, in evolutionary terms, runs through the medial region of the brain stem and comprises the paleospinothalamic, spinoreticular, spinomesencephalic, spinoparabrachio-amygdaloid, spinoparabrachio-hypothalamic, and spinohypothalamic bundles (Millan, 1999; Almeida et al., 2004). The more recent pathway is located in the lateral region of the brain and is formed by the neospinothalamic and spinocervical bundles (Millan, 1999; Almeida et al., 2004).

The paleospinothalamic and neospinothalamic pathways form the spinothalamic tract, which is mainly involved in the sensorial aspects, discriminating features such as duration, temporal pattern, location and intensity of pain, temperature, touch and itch-related information (Millan, 1999; Almeida et al., 2004). The projections come from laminae I, II, IV, V, VI, VII, VIII, and X to different nuclei of the thalamus. The neospinothalamic pathway projects to the lateral complex of the thalamus and seems to be involved in the sensory-discriminative component of pain, while the paleospinothalamic pathway projects to the posterior medial and intralaminar complex of the thalamus and is more involved with motivational-affective aspects (Almeida et al., 2004).

The spinoparabrachial tract represents a direct nociceptive pathway, with projections to the parabrachial nucleus (PN) (Millan, 1999; Almeida et al., 2004). Other pathways have direct or indirect projections to the PN. The neurons originate in laminae I and II. This tract seems to be involved in visceral, inflammatory and thermal nociceptive processing and has projections to limbic structures like the amygdala, and also to the hypothalamus. It is also involved in autonomic, motivational, affective and neuroendocrine responses to pain (Millan, 1999; Almeida et al., 2004).

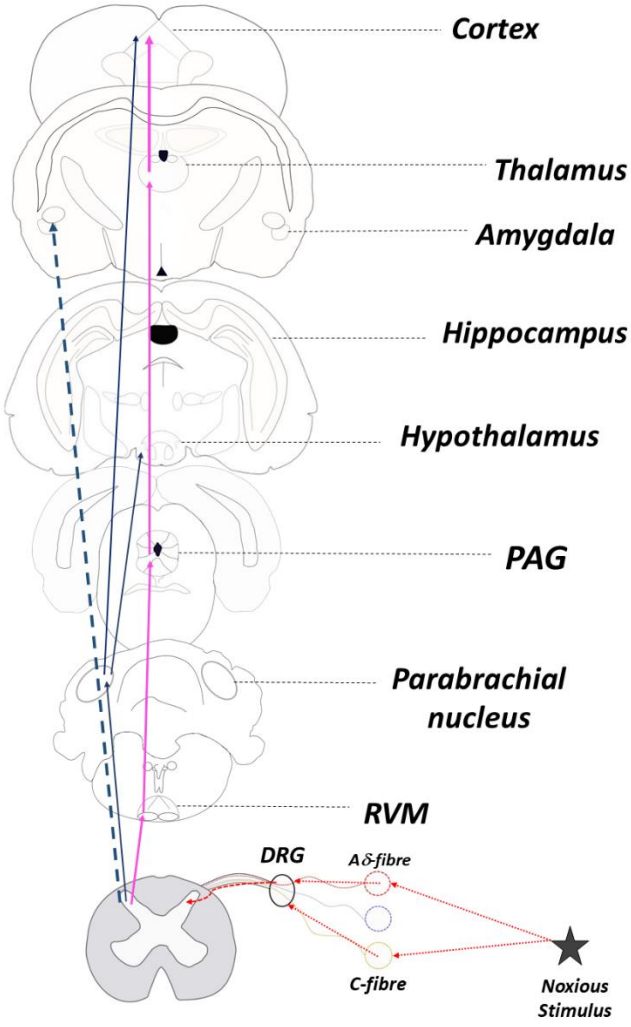


Figure 1.1: Graphical representation of two of the main ascending pain pathways, spinothalamic and spinoparabrachial. The nociceptive stimulus generates a receptor and action potential that is relayed through the primary afferent fibres through the DRG to dorsal horn of the spinal cord (dashed red lines, bottom right). The spinothalamic pathway (in pink) sends projections to the thalamus via PAG, and from there to the cortex. This tract is involved in sensory-discriminative aspects of pain. The spinoparabrachial pathway (in blue) is important in the cognitive-affective aspects of pain, and projects to the amygdala (dashed blue line) and hypothalamus and cortex (solid blue lines) through the PN. DRG, dorsal root ganglia; PAG, periaqueductal grey; PN, parabrachial nucleus; RVM, rostral ventromedial medulla.

1.2.2 Descending Pain Pathway

The nociceptive information sent through the ascending pathways is processed, and potentially modified – reduced or amplified – by supraspinal structures. The reduction of nociception resulting from supraspinal modulation is known as descending inhibition and the enhancement of nociceptive responses is known as descending facilitation. Descending pathways originate from cerebral structures, and modulate the nociceptive response through the control of neurotransmission in the dorsal horn of the spinal cord, which includes the terminals of primary afferent neurons and the secondary sensory neurons (Millan, 1999, 2002).

These supraspinal regions with direct projections which are involved in the modulation of nociceptive response include the RVM, PAG, PN, hypothalamus, and cerebral cortex (Millan, 2002). The PAG receives input projections from the central nucleus of the amygdala (CeA) (Pittman et al., 1981; da Costa Gomez and Behbehani, 1995; Da Costa Gomez et al., 1996), while the amygdala receives inputs from the prefrontal cortex (PFC; McDonald, 1987; Brinley-Reed et al., 1995). Additionally, the CeA directly projects to the PN (Neugebauer et al., 2004), that will then project to the spinal cord (Kuroda et al., 1987; Ma and Peschanski, 1988). The thalamus also has projections to the PAG (Vasilenko and Eliseeva, 1980; Barbaresi et al., 1982). Finally, the PAG has direct projections to the RVM (Millan, 2002). Therefore, these regions are important sites for the activation and/or modulation of the descending inhibitory pathway that projects to the dorsal horn of the spinal cord and modulates nociceptive response (Millan, 2002).

Descending facilitation is an increased spinal dorsal horn neuronal response to noxious stimuli (Zhuo, 2017). The cerebral regions involved in descending facilitation are the same as those responsible for the inhibitory actions described above. Therefore, the switch between activation and inhibition is mediated by differences in neurotransmitter activity (Rahman et al., 2009; De Felice and Ossipov, 2016). In certain circumstances, the balance between inhibition and facilitation can be affected and the correct modulation of these systems can be disturbed. This imbalance is one of the possible causes for a state of chronic pain (see section 1.2.3).

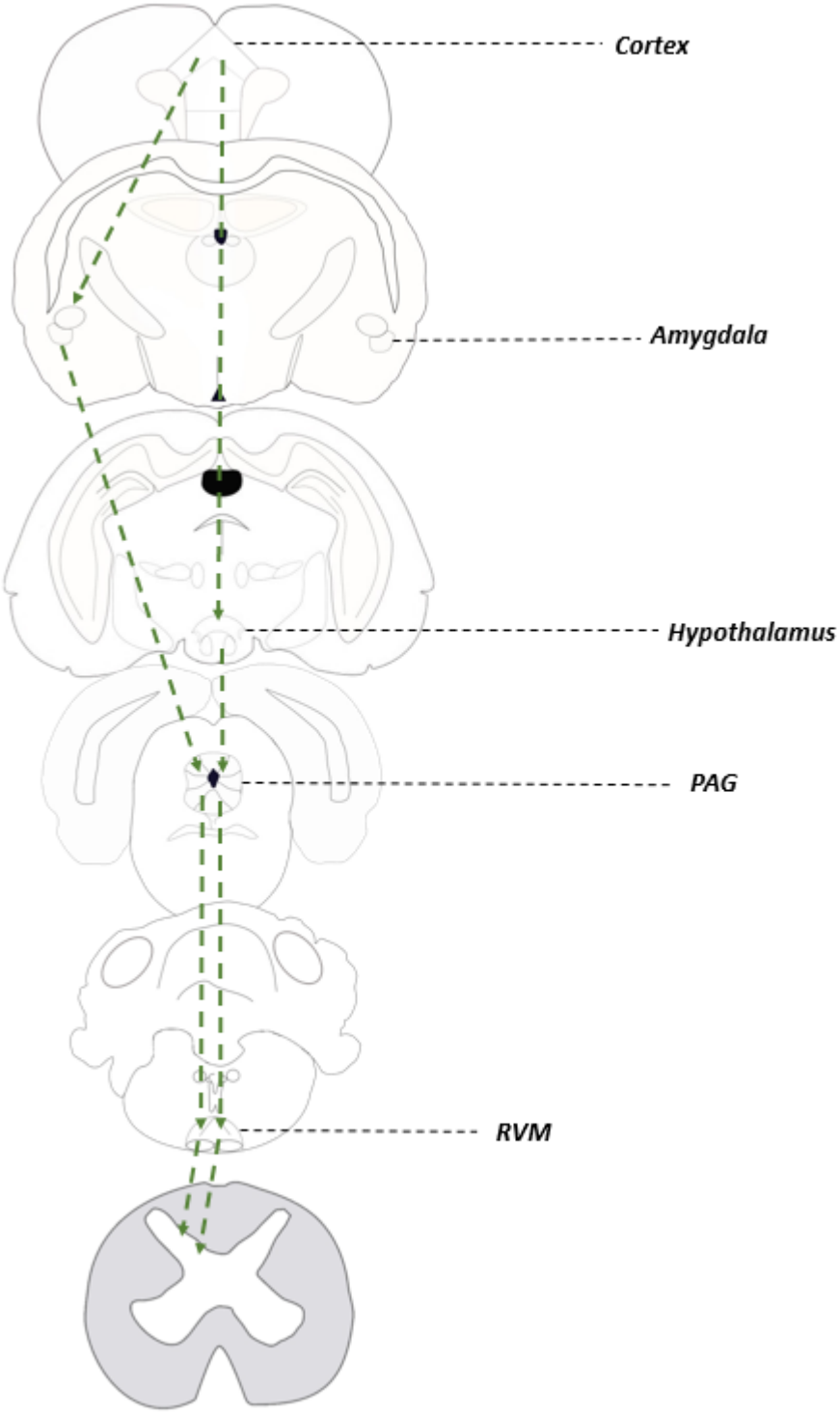


Figure 1.2: Graphical representation of the descending pain pathway. It originates from higher regions including the cortex, amygdala, and hypothalamus that project to the PAG and RVM, which in turn projection to the dorsal horn of the spinal cord. PAG, periaqueductal grey; RVM rostral ventromedial medulla.

1.2.3 Pathophysiology of chronic pain

The brain receives several signals triggered by different stimuli (i.e. mechanical, chemical, thermal, biological) through different sensory systems (i.e. visual, auditory, gustatory, olfactory, somatosensory and vestibular). These stimuli are transduced to receptor potentials and then in afferent action potentials that will be sent to specific supraspinal areas. Normally, there is a balance in the inhibition or facilitation of pain signals. Chronic pain can be a consequence of a disturbance in this equilibrium; it can be a consequence of the activation of descending facilitation, impaired descending inhibition, or abnormal peripheral or central sensitisation such as action potential windup in which the repeated stimulation of the dorsal root afferents can elicit a progressive increase in the number of action potentials generated by second order neurons in the dorsal horn (Cross, 1994; Baranauskas and Nistri, 1998).

In normal conditions, descending inhibition and facilitation are in equilibrium, which can be modified under certain pathological conditions, resulting in chronic inflammatory, neuropathic or visceral pain (Cross, 1994; Pertovaara, 1998; Burgess et al., 2002; Ossipov et al., 2014; Zhuo, 2017). There is no anatomical difference between descending inhibitory and facilitatory pathways, and the accurate activation of each is mediated by different receptors or isoforms of receptors (Rahman et al., 2009; De Felice and Ossipov, 2016).

Peripheral sensitization is the reduction in the threshold of nociceptors caused by local inflammatory substances such as bradykinin and prostaglandins that are released after a trauma (Curatolo et al., 2006). These mediators induce changes in the normal threshold response of primary afferent fibres and result in increased responsiveness to noxious (hyperalgesia) or innocuous (allodynia) stimuli. This phenomenon results in an amplified nociceptive input to the spinal cord, which in turn may cause a reversible increase in neuronal activity in the dorsal horn, known as central sensitization (Schwartzman et al., 2001; Curatolo et al., 2006; Woolf, 2011; Spiegel et al., 2017). As the tissue recovers, peripheral and central sensitization normally decline, and pain thresholds return to normal state. However, in some situations, the afferent fibres or central pathways get damaged as a complication of pathological conditions or physical rupture. In this situation, thresholds may not return to normal, a condition referred as neuropathic pain (Schwartzman et al., 2001; Campbell and Meyer, 2006; Woolf, 2011; Spiegel et al., 2017).

1.3 Neurobiology of fear and anxiety

Fear and anxiety are protective states that are associated with defensive behavioural responses. They have evolutionary importance since they serve as an alert to potential harmful or dangerous stimuli/situations and, therefore, ensure survival and safety. Upon exposure to a fearful stimulus, a chain of measurable behavioural, physiological, hormonal and autonomic responses is elicited. Because fear and anxiety are well-conserved across species, scientists have developed several correlated animal models for the study of these phenomena, which have facilitated substantial knowledge about the brain regions, cellular mechanisms and neurocircuitry involved in fear and anxiety responses. In fact, most of what we know about fear comes from studies using classical (or Pavlovian) fear conditioning: in this paradigm, a previously neutral stimulus (i.e. a stimulus that does not elicit any fear/anxiety response *per se;* e.g. a tone) is paired to an unconditioned stimulus (US), which evokes innate fear responses (e.g. predator odour or footshock). The innocuous stimulus, following association with the US, is then able, when presented alone, to evoke the same behavioural and physiological fear-related reactions, and as a result is then called conditioned stimulus (CS). Due to its simplicity and broad application, fear conditioning has been widely used. Importantly, the paradigm itself has a learning process involved – the association of the US to the CS (associative learning) – and can also be applied to examine learning and memory processes.

The brain regions and neuromodulatory system for fear and anxiety have great overlap, and the behavioural output of the endocrine, autonomic and physiological responses are greatly similar. In fact, part of what is known about the anxiety-related neurocircuitry is an extrapolation of fear-based investigations, and much still needs to be elucidated. Anxiety is emotionally more complex due to its nature: while fear is elicited upon actual and tangible threats, while anxiety is triggered by the anticipation of danger.

1.3.1 Neurocircuitry of fear

The neurocircuitry of fear and anxiety is the focus of numerous research articles and reviews. An overview of the circuitry is described below, with an emphasis on the regions that are most relevant to this thesis.

Once the stimulus is perceived by one of the sensorial systems (i.e. smell of a predator – olfactory system), the information is sent to the thalamus (TH), primary sensory cortices,

and association cortices (Figure 1.3). The association cortices have excitatory outputs to the lateral central amygdala (lCeA). Additionally, the thalamus projects to the lateral amygdala (LA) and to the basolateral amygdala (BLA). The LA also receives inputs from the primary sensory cortices which is conveyed to the BLA. The BLA sends excitatory projections to the ventral hippocampus (VH), prelimbic (PL) and infralimbic (IF) cortices and to the medial central amygdala (mCeA). Another indirect projection from the BLA to the mCeA through the intercalated cells of the amygdala (ITC) is also reported. Then, the mCeA sends inhibitory projections to the PAG and to the hypothalamus, promoting the behavioural and physiological responses to fear. PAG is known to modulate freezing behaviour and the hypothalamus is involved in the endocrine and physiological fear outcomes.

Therefore, the amygdala, the hippocampus, and the PAG are key regions in fear acquisition and/or expression. The specific role of these regions in fear and anxiety are going to be further explored in the sections that follow. Special attention will be given to the amygdala, because four of the six studies described in this thesis are focused on two subnuclei of the amygdala – BLA and CeA.

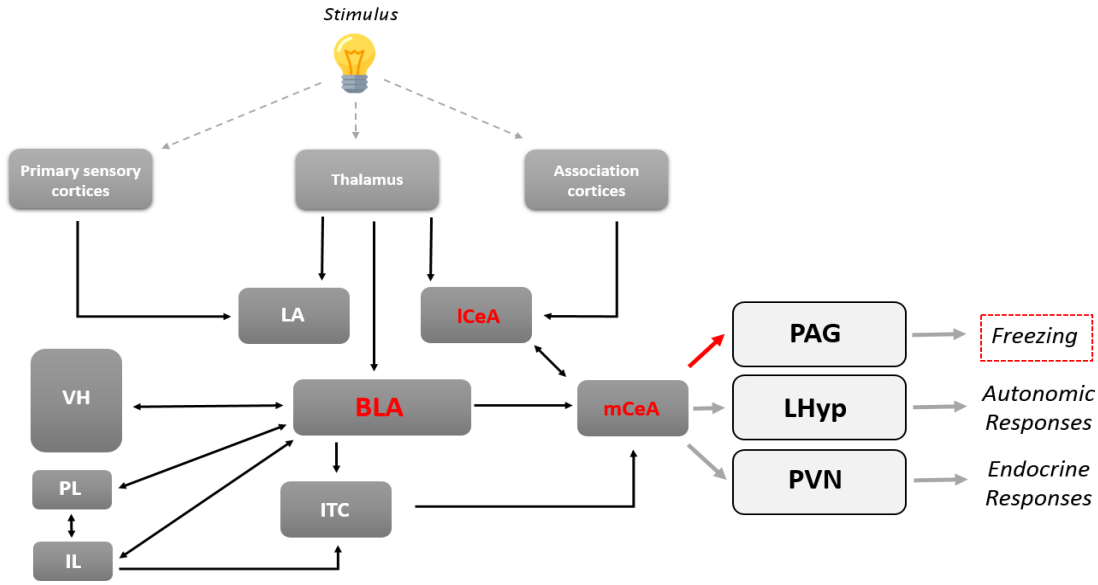


Figure 1.3: Graphical representation of fear neurocircuitry. In red, the amygdalar regions involved in fear and anxiety responses. LA, lateral amygdala; lCeA, lateral central amygdala; BLA, basolateral amygdala; mCeA, medial central amygdala; ITC, intercalated cells of the amygdala; VH, ventral hippocampus; PL, prelimbic cortex; IL, infralimbic cortex; PAG, periaqueductal grey; LHyp, lateral hypothalamus; PVN, paraventricular nucleus of the thalamus.

1.3.1.1 **Amygdala -** ***Neuroanatomy and neurophysiology***

The amygdala is an almond shaped structure localised in the temporal lobe. Burdach was the first scientist to identify and describe this structure (although his description only included what we today know as the basolateral complex) back in the 19th century (Sah et al., 2003). Subsequently, Paul McLean introduced the "visceral brain" idea and the concept of a limbic system to structures believed to be involved in emotion and/or emotional responses. To these structures, he included the amygdala (McLean, 1949). Later, studies by Kluver and Bucy demonstrated that lesions of the medial temporal lobe of monkeys impaired emotional responding (Klüver, H., & Bucy, 1937, 1939). Finally, with the experiments of Weiskrantz in which he restricted the lesions to the amygdaloid complex, the importance of the amygdala in emotional processing was defined (Weiskrantz, 1956). Afterwards, studies using classical and instrumental conditioning cemented the role of the amygdala and its neurocircuitry not only in fear, but also anxiety and memory processing. Very interestingly, Adolphs *et al* reported in 1994 the case of a woman identified only as S.M. who suffered from Urbach-Wiethe disease, a condition that causes a nearly complete bilateral destruction of the amygdala while sparing hippocampus and other neocortical regions. Thanks to the help of S.M., it was possible to observe the role of the amygdaloid complex in emotional face recognition and endorse the role of the region in emotion processing.

The amygdala is a broad and heterogeneous region that comprises ~13 nuclei that differ in cytostructure, embryonic origin, histochemistry and afferent/efferent connections. There are numerous reviews on the neuroanatomical division of the amygdala, but the most common nomenclature is the one introduced by Price et al (1987) in which the amygdala is divided into three regions: (1) basolateral (BLA), (2) cortical, (3) and centromedial (CeA) (see Figure 1.4 for details of the subnuclei included in each of the groups mentioned above). However, some authors, based on anatomical studies from Alheid and Heimer et al (1988) argue that regions like the bed nucleus of the stria terminata (BNST) and some regions of the substantia inominata should be included in the amygdaloid complex, due to its similarity in origin and efferent connections. More specifically, they argue that these regions are an extension of the centromedial complex and should therefore be recognised as "extended amygdala".

Another recent neuroanatomical organisation of the amygdala was proposed by Swanson and Petrovich (Swanson and Petrovich, 1998). They took into consideration the

developmental origin of each region and subdivided the amygdala into four regions: (1) frontotemporal, which incorporates regions with cortical-like neurons (i.e. cells that receive similar afferent connections and contain similar cytoarchitecture to cortical neurons), (2) autonomic, which includes regions involved in autonomic control and with striatum-like neurons, and (3) main olfactory and (4) accessory olfactory, which are targets of olfactory projections. Swanson-Petrovich (SP) organisation fits well with the widely-used Price (Pr) organisation: frontotemporal (SP) correlates with the basolateral complex (Pr), autonomic (SP) with the centromedial complex (Pr), and the main and accessory groups (SP) with the cortical complex (Pr). Therefore, I decided to use the most common nomenclature proposed by Price for this thesis.

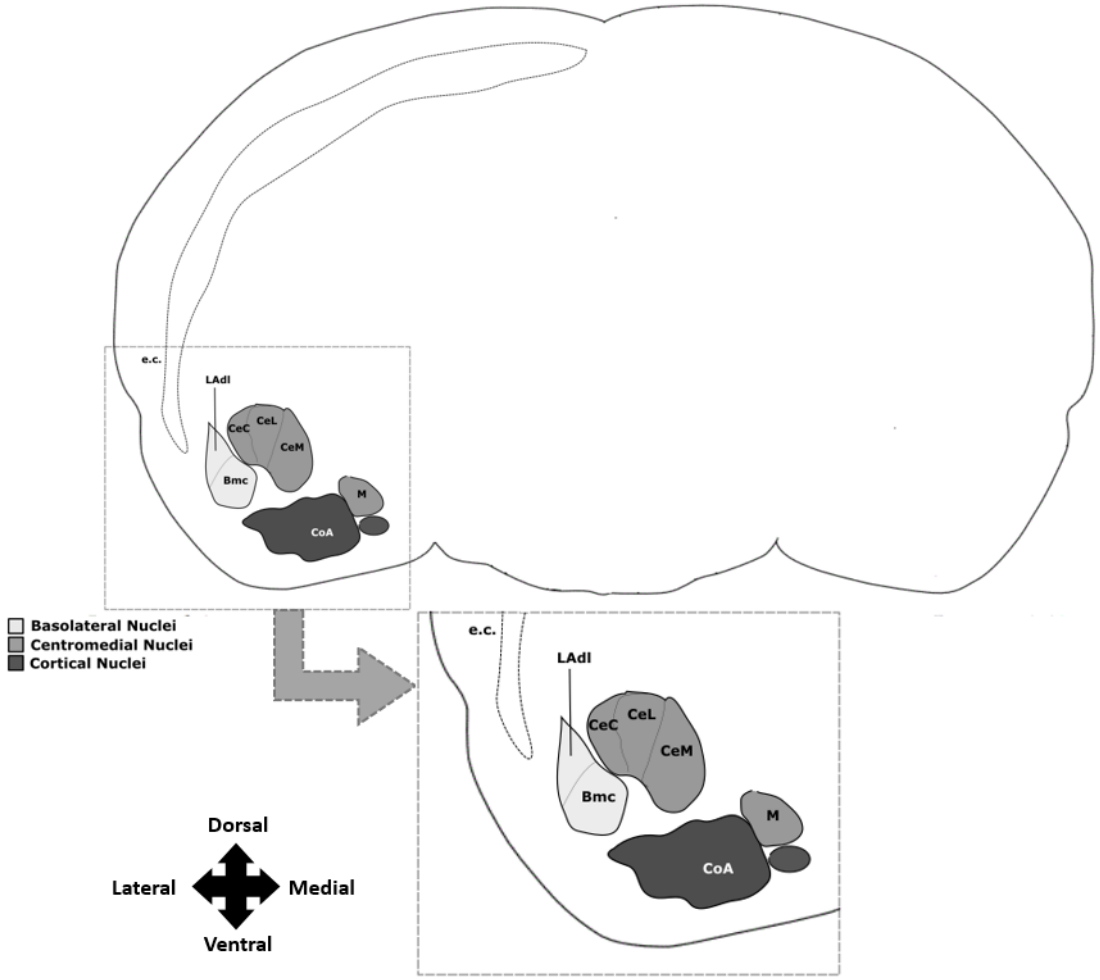


Figure 1.4: Graphical representation of the anatomical location of the amygdala (dashed square) and the subdivision of the amygdala. LAdl, dorsolateral lateral amygdala; Bmc, basal amygdala; CeC, capsular central amygdala; CeL, lateral central amygdala; CeM, medial central amygdala; CoA, anterior cortical nucleus.

Most of the cells found in the BLA (~70% according to Sah et al, 2003) are pyramidal-like (or projection) neurons. They are glutamatergic neurons, which form the majority of the BLA. The second main group of cells in the BLA is the spiny cells. These are GABAergic interneurons responsible for the local flow of information. On the other hand, the predominant cell type in the CeA is the medium spinal neurons, similar to the spinal neurons found in the striatum. These are GABAergic neurons. Thus, while the projections of the BLA are mainly glutamatergic, projections from the CeA are mainly GABAergic.

As mentioned before, the amygdala is part of the limbic system. Therefore, it has been studied extensively for its role in emotional responses and, consequently, is the subject of multiple reviews on its role in fear (Deutch and Charney, 1996; Charney et al., 1998; LeDoux, 2000, 2007, 2014; Davis and Whalen, 2001; Radulovic and Spiess, 2001; Paré et al., 2004; Shin and Liberzon, 2010; Herry et al., 2010a; Orsini and Maren, 2012; Lalumiere, 2014; Tovote et al., 2015; Sah, 2017; Garcia, 2017) and anxiety (Gilpin et al., 2015; Linsambarth et al., 2017). Several studies show that lesions or inactivation of different nuclei of the amygdala impair the expression of fear behaviour in rodents (see Table 1.1). Lesions of the amygdala in humans also disrupt fear responses (Adolphs et al., 1994; Anderson and Phelps, 2001).

The BLA is considered the hub for fear/anxiety responses due to its central position in the circuitry (see Figure 1.3). It receives important inputs from different regions (i.e. LA, thalamus, hippocampus, and PFC) and sends projections to the CeA which transmits the information to the PAG (further discussed in section 1.3.3) and hypothalamus. These regions are responsible for the final behavioural and physiological outcome. Further studies confirmed the importance of the BLA in fear and anxiety. The inactivation of the BLA and the VH impaired fear expression and extinction in rats (Malin and McGaugh, 2006; Sierra-Mercado et al., 2011b). Additionally, synaptic plasticity within the BLA was shown to be crucial for fear memory formation (Maren, 1996; Ressler and Maren, 2019). Furthermore, other studies applying optogenetic (Huff et al., 2013; Lalumiere, 2014) and genetic (Pape and Stork, 2006; Haubensak et al., 2010) methods confirmed the key modulatory role of the BLA in fear expression. Recently, the circuitry behind fear expression has been extensively investigated (Davis and Reijmers, 2018). Hence, neuronal networks linking the BLA with other brain regions were revealed to be of great importance for anxiety and fear responses, especially the BLA-Hippocampus (Sparta et al., 2014; Yang and Wang, 2017; Wahlstrom et al., 2018a) and BLA-mPFC (McGarry and Carter, 2017; Bloodgood et al., 2018; Uliana

et al., 2018; Lingawi et al., 2019) pathways. The BLA has a similar vital role in anxiety modulation (Bruchas et al., 2009; Etkin et al., 2009; Roozendaal et al., 2009; Knoll et al., 2011; Babaev et al., 2018). Tye et al (2011) have reported that optogenetic activation of BLA terminals in the CeA results in a robust anxiolytic effect. The photoinhibition of the projections from the BLA to the VH also had anxiolytic effects, while its activation increased anxiety (Felix-Ortiz et al., 2013). Noradrenergic release into the BLA was shown to have anxiogenic effects (McCall et al., 2017), and chemogenetic and optogenetic activation of β_2-adrenergic receptors also increases innate and social anxiety (Siuda et al., 2016). Importantly, the glutamatergic (Zimmerman and Maren, 2010; Li and Rainnie, 2014) , GABAergic (Makkar et al., 2010b; Babaev et al., 2018), dopaminergic (Pezze et al., 2005; de Oliveira et al., 2014; Li and Rainnie, 2014; Lee et al., 2017), serotoninergic (Bauer, 2015a), noradrenergic (Roozendaal et al., 2006), endocannabinoid (Lutz et al., 2015; Lisboa et al., 2017; Patel et al., 2017; Morena et al., 2018a), and opioid (Knoll et al., 2011; Nummenmaa and Tuominen, 2018) systems in the BLA were revealed to be involved in fear and anxiety responses.

For a long time, the CeA was only seen as an output subdivision of the amygdala, because of the outcomes of its lesion or inactivation on behavioural and autonomic responses to fear (table 1.1), which had similar outcomes to PAG and hypothalamus lesions. These studies lead to the notion that CeA mediates fear through downstream projections to these regions. However, recent studies have revealed a more significant role for the CeA in fear and anxiety both in rodents (Ciocchi et al., 2010a; Carvalho et al., 2015; Cai et al., 2018) and in monkeys (Kalin et al., 2004). Notably, instead of being a homogeneous structure, the CeA was revealed to have several subdivisions with different cytoarchitectures, functions and inputs – while its medial portion (medial central nucleus of the amygdala; mCeA) is the main source of output projections, the lateral division (lateral central nucleus of the amygdala; lCeA) is comprised of inhibitory circuits (Keifer et al., 2015a). This discovery was very important because it triggered more focused investigations on the modulation of each individual subdivision of the CeA. For example, Ciocchi et al (2010) have demonstrated that the optogenetic activation of the mCeA resulted in freezing expression and muscimol administration into the lCeA, but into mCeA or entire CeA, resulted in unconditioned freezing. Moreover, the inactivation of lCeA during fear conditioning lead to fear expression impairment and the inactivation of mCeA and entire CeA 24 hours after conditioning resulted in freezing expression deficits. Additional to its important role in fear

expression, CeA was also shown to be involved in anxiety-related responses (Davis et al., 2010; Lyons and Thiele, 2010; Gilpin et al., 2015; Fox and Shackman, 2019). Lesions of the CeA were shown to attenuate stress-induced anxiety behaviour (Ventura-Silva et al., 2013). Moreover, as previously reported, the activation of the BLA-CeA pathway was shown to reduce anxiety (Tye et al., 2011).

The LA was also extensively investigated due to its strong connections to primary sensorial cortices. Thus, the LA is an important region in tone and sound-based models for associative conditioning. Recently, two studies using optogenetic tools confirmed the important role of the LA in classical fear conditioning. The photoactivation of LA neurons simultaneously to the presentation of a CS could be used as a substitute for the footshock US, resulting in conditioned freezing (Johansen et al., 2010). Another study showed that brief photoactivation of LA axonal terminals from the auditory thalamus and the auditory cortex can substitute for a tone CS when paired with footshock, resulting in conditioned freezing and synaptic potentiation (Nabavi et al., 2014).

In situ hybridization has shown that the amygdala has a rich distribution of dopaminergic receptor 2 (D_2; Meador-Woodruff et al., 1991a, 1991b). There are a good number of studies supporting the notion that the dopaminergic system in the amygdala is involved fear and anxiety expression (for a review see Brandão and Coimbra, 2018). For instance, antagonists at D_2 reduce the acquisition and expression of Pavlovian fear conditioning. Moreover, an intra-BLA injection of the D_2 antagonist SCH23390 inhibited fear-potentiated startle (de Oliveira et al., 2011a) and attenuates conditioned fear (Nader and LeDoux, 1999). Infusions of sulpiride, a dopaminergic antagonist, before acquisition or before both acquisition and retention testing also significantly attenuated contextual conditioned freezing during the retention test 24 h later (de Oliveira et al., 2006; Oliveira et al., 2009). Thus, these findings emphasize the importance of the dopaminergic system in the formation and/or consolidation of fear memories.

The amygdala receives serotoninergic innervation and serotoninergic receptors type 2 and 3 were shown to be expressed in the region (Pazos and Palacios, 1985; Tecott et al., 1993; Yilmazer-Hanke et al., 2003; Hensler, 2006; Smith and Porrino, 2008; Asan et al., 2013). The pharmacological manipulations of serotonin transmission in the amygdala had effects on anxiety (Menard and Treit, 1999; Lowry et al., 2005; Christianson et al., 2010; for a review see Asan et al., 2013) and fear-related behaviour (for a review see Bauer,

2015b). For instance, microdialysis studies suggest that both CS and US presentations are capable of enhancing 5-HT release in the BLA, with increased 5-HT in response to inescapable shocks (Amat et al., 1998) and fear memory retrieval (Zanoveli et al., 2009). Moreover, depletion of 5-HT by 5, 7-dihydroxytryptamine injections in the amygdala had reduced acquisition of fear during conditioning and recall on subsequent testing days (Johnson et al., 2015).

In summary, the amygdaloid complex and its divisions have a key modulatory role in fear and anxiety responses. Hence, it is important to further explore their neurocircuitry and neurophysiology in order to advance our understanding of emotional processing in the brain and identify new therapeutic targets for anxiety and fear disorders.

Table 1.1: Studies showing the effects of lesion or inactivation of the amygdala subnuclei on fear conditioning responses.

Lesion/inactivation	Region	Type of lesion/inactivation	Animal	Fear conditioning protocol	Outcome	Reference
Lesion and inactivation	BLA	Lesion: NMDA injections Inactivation: Muscimol injection	Rats	Unconditioned footshock	Neither neurotoxic BLA lesions nor temporary inactivation of the BLA during overtraining prevented the inflation effect.	Rabinak and Maren, 2008
Lesion and inactivation	BLA and CeA	Lesion: NMDA injections Inactivation: Muscimol injection	Rats - Long Evans	Auditory and contextual conditioning	Rats with pre-training CeA lesions (whether alone or in combination with BLA lesions) did not acquire conditional freezing to either the conditioning context or an auditory conditional stimulus after extensive overtraining. Similarly, post-training lesions of the CEA or BLA prevented the expression of overtrained fear. Muscimol infusions into the CeA prevented both the acquisition and the expression of overtrained fear.	Zimmerman et al., 2007
Lesion and inactivation	BLA and LA* *described as frontotemporal amygdala, which comprises these two subdivisions	Lesion: NMDA injections Inactivation: Muscimol injection	Rats	Auditory fear conditioning (tone paired with footshock)	Lesions of the frontotemporal region of the amygdala, which includes lateral and basal nuclei, cause a loss of conditional fear responses, such as freezing. Fear memory is abolished if BLA and LA is inactivated by muscimol during the inflation treatment with strong shocks.	Fanselow and Gale, 2006
Lesion	BLA, LA and CeA	Electrolytic lesions	Rats	Auditory and contextual conditioning	LA and CeA lesions attenuated freezing to both contextual and auditory conditional stimuli. Lesions of the basal nuclei produced deficits in contextual and auditory fear conditioning only when the damage extended into the anterior divisions of the basal nuclei	Goosens and Maren, 2001

Lesion	BLA	Olney's lesions - NMDA receptor antagonist neurotoxicity (NAN)	Rats - Long Evans	Contextual conditioning and Auditory fear conditioning (tone + footshock)	BLA lesions before conditioning reduced freezing (1 or 25 conditioning trials). Post conditioning BLA lesions extinguished the memory for Pavlovian fear (1 or 75 trials); Results in both contextual and auditory conditioning	Maren et al., 1999
Lesion	BLA	Not reported	Not reported	Olfactory fear conditioning	Pretraining excitotoxic lesions of the BLA abolished immediate postshock freezing, conditioned freezing to an olfactory CS, and conditioned freezing to the training context. Excitotoxic lesions of the BLA produced either 1 day or 15 days after olfactory fear conditioning abolished both odor-elicited and contextual freezing.	Couseans and Otto, 1998
Lesion	BLA	NMDA injections	Rats	Auditory fear conditioning (tone paired with footshock)	BLA-lesioned rats displayed robust freezing deficits across both short-term (24hr) and long-term (16 months) tests.	Gale et al., 2004
Lesion	BLA	NMDA injections	Rats - Long Evans	Context Conditioning	Post-training BLA lesions resulted in strong deficits in contextual freezing expression. Overtraining does not affect the magnitude of these deficits. Similarly, overtraining did not influence the level of reacquisition obtained by rats with post-training BLA lesions after 10 reacquisition trials. A similar pattern of results was observed in rats with pre-training BLA lesions	Maren, 1998
Lesion	BLA	NMDA injections	Rats - Long Evans	Auditory and contextual conditioning	There were severe effects of post-training BLA lesions on the expression of conditional freezing even after extensive presurgical overtraining (25–75 trials). Moreover, there was no evidence for sparing of fear memory (i.e., savings) in these rats.	Maren, 2001

Lesion	BLA	NMDA injections	Rats - Long Evans	Auditory and contextual conditioning	Pretraining BLA lesions yielded severe deficits in the acquisition of conditional freezing in rats trained with either 1 or 25 conditioning trials. However, extensive overtraining (50 or 75 trials) mitigated deficits in conditional freezing in the contextual but not acoustic protocol. Post-training BLA lesions eradicated the memory for Pavlovian fear in rats trained with either 1 or 75 trials; this deficit was not modality-specific	Maren, 1999
Lesion	BLA	NMDA injections	Rats - Long Evans	Auditory and contextual conditioning	BLA lesions abolished conditional freezing to both the contextual and acoustic conditional stimuli. Reacquisition training elevated levels of freezing in rats with BLA lesions but did not reduce the magnitude of their deficit in relation to that of controls.	Maren et al., 1996a
Lesion	BLA	APV injection (NMDA antagonist)	Rats - Long Evans	Context Conditioning	APV infusion into the basolateral amygdala (BLA), before training, disrupted the acquisition of contextual fear. APV produced a disruption of both the acquisition and expression of contextual fear. This blockade of contextual fear was not state dependent, not due to a shift in footshock sensitivity, and not the result of increased motor activity in APV-treated rats. Fear conditioning was not affected by a post-training APV infusion into the BLA	Maren et al., 1996b
Lesion	CeA	Electrolytic lesions with anodal currents	Rats - Wistar Rats	Contextual conditioning	Lesioning of the CEA completely abolished the bradycardiac response. Immobility behaviour was slightly diminished.	Roozendaal et al., 1990
Lesion	CeA	Electrolytic lesions with anodal currents	Rats - Wistar Rats	Contextual conditioning	However, CEA lesioning attenuated the bradycardiac response and the immobility behavior during the late part of the test.	Roozendaal et al., 1991

Lesion	CeA	Electrolytic lesions	Rats	Contextual conditioning	CeA-lesioned rats exhibit significant less freezing and USV than non-lesioned counterparts when CS was presented.	Choi and Brown, 2003
Lesion	Amygdala and Hippocampus	Electrolytic lesions with anodal currents	Rats - Sprague-Dawley	Contextual conditioning and Auditory fear conditioning (tone + footshock)	Pre-conditioning lesions of the amygdala disrupted conditioning of fear responses to both the cue and the context. Lesions of the hippocampus interfered with conditioned responses to the context only	Phillips and LeDoux, 1992
Lesion	Amygdala, Hippocampus and PAG	Electrolytic lesions with anodal currents	Female Rats - Long Evans	Contextual conditioning	Rats with amygdala or vlPAG lesions exhibited a significant attenuation in freezing both immediately and 24 hr after the shocks. Animals with hippocampal lesions displayed a marked deficit in freezing 24 hr after the shock	Kim et al., 1993
Lesion	BLA and CeA	Electrolytic lesions with anodal currents	Rats - Long Evans	Contextual conditioning	Lesion in both BLA and CeA reduced amount of freezing. Also, lesions in both nuclei disrupted FCA.	Helmstetter, 1992
Lesion	BLA and CeA	Ibotenic acid	Rats	Conditioned punishment and suppression	Rats with lesions of the CeA exhibited reduction in the suppression of behaviour elicited by a conditioned fear stimulus, but were simultaneously able to direct their actions to avoid further presentations of this aversive stimulus. In contrast, animals with lesions of the BLA were unable to avoid the conditioned aversive stimulus by their choice behaviour, but exhibited normal conditioned suppression to this stimulus.	Killcross et al., 1997

Lesion	BLA and CeA	BLA: NMDA injections CeA: Ibotenic acid and eletrolytic	Rats	Auditory and contextual conditioning	Postshock freezing and USV responses were significantly impaired in BLA-lesioned animals, whereas CeA-lesioned animals exhibited only mild deficits. Similarly, conditioned fear responses assessed 24 hr after training were severely reduced in BLA-lesioned animals but not in CeA-lesioned animals. In contrast to ibotenic lesions of the CeA, small electrolytic lesions of the CeA strongly affected both postshock and conditioned freezing and USV.	Koo et al., 2004
Lesion	BLA, CeA, LA, acessory amygdala, medial amygdala and entire amygdala	Eletrolytic lesions	Rats - Sprague-Dawley	Auditory Conditioning	Animals that received lesions in the LA, CeA or the entire amygdala, were dramatically impaired, whereas the other lesions had little effect.	Nader et al., 2001
Lesion	CeA and BLA	Ibotenic acid and electrolytic lesions	Rats - Sprague-Dawley	Acoustic Startle reflex	Lesions of the CeA blocked fear-potentiated startle to both auditory and visual CSs. Similarly, pre- or post- training electrolytic or NMDA-induced lesions of the BLA disrupted fear-potentiated startle to both CS modalities.	Campeau and Davis, 1995
Lesion	LA and BLA	N-methyl-D-aspartate	Rats - Sprague-Dawley	Fear-potentiated startle	Lesions before and after conditioning completely blocked fear-potentiated startle (increased acoustic startle in the presence of a light previously paired with footshock)	Sananes and Davis, 1992
Inactivation	BLA	AP5 and AP7 (NMDA antagonists)	Rats	Fear-potentiated startle	NMDA antagonists infused into the amygdala block the acquisition but the expression of fear conditioning	Miserendino et al., 1990

Inactivation	BLA	injection of muscimol	Rats - Long Evans	Contextual conditioning	Inactivation of BLA before test session showed a significantly attenuated fear response, but resulted in a much smaller decrement in conditional fear when muscimol was injected prior to training (conditioning)	Helmstetter and Bellgowan, 1994
Inactivation	BLA	Muscimol inactivation	Rats - Wistar	Plus-maze discriminative avoidance task	Pre-training muscimol prevented memory retention, but did not alter innate fear. Post-training muscimol impaired consolidation, inducing increased percent in aversive arm exploration in the test session. Pre-testing muscimol did not affect retrieval.	Ribeiro et al., 2011
Inactivation	BLA and CeA	AP5 injections	Female Rats - Long Evans	Contextual conditioning	Administration of AP5 to the basolateral nucleus prevented acquisition of fear. Central nucleus infusions had no effect.	Fanselow and Kim, 1994
Inactivation	BLA and CeA	NBQX inactivation	Rats - Long Evans	Auditory fear conditioning (tone paired with footshock)	NBQX infusions into the BLA impaired the acquisition of auditory fear conditioning with an inflation-magnitude US, indicating that the amygdala is required for associative learning with intense USs.	Rabinak et al., 2009
Inactivation	BLA and CeA	APV injection (NMDA antagonist)	Rats	Auditory and contextual conditioning	BLA or CeA blockade during fear conditioning impaired both auditory and contextual fear conditioning. Some conditioned fear was exhibited by rats infused with APV into the CeA but not the BLA.	Goosens and Maren, 2003
Inactivation	BLA and LA	Muscimol inactivation	Rats - Sprague-Dawley	Auditory Conditioning	Pre-training, but not post-training, infusions eliminated acquisition of fear memory.	Wilensky et al., 1999

Inactivation	BLA, CeA and BNST	NBQX	Rats - Sprague-Dawley	Fear and Light-potentiated startle reflex	Infusions into the central nucleus of the amygdala blocked fear-potentiated but not light-enhanced startle, and infusions into the bed nucleus of the stria terminalis blocked light-enhanced but not fear-potentiated startle. Infusions into the basolateral amygdala disrupted both phenomena.	Walker and Davis, 1997
Inactivation	CeA and LA	Muscimol inactivation	Rats - Sprague-Dawley	Auditory fear conditioning (tone paired with footshock)	CeA is involved not only in the expression but also the acquisition of fear conditioning. Also, inhibition of protein synthesis in the CeA after training impairs fear memory consolidation.	Wilensky et al., 2006
Inactivation	LA and BLA	injection of muscimol	Rats - Sprague-Dawley	Contextual conditioning and Auditory fear conditioning (tone + footshock)	Inactivation of LA and BLA before training session disrupt fear learning and expression. Results in both contextual and auditory conditioning	Muller et al., 1997
Inactivation	BLA and CeA	Injection of muscimol	Rats - Wistar	Contextual conditioning	BLA and CEA inactivation change the expression of conditioned fear, in a paradigm using the context as the conditioned stimulus (CS). These changes are correlated to the innate anxiety levels of the animals.	Nobre, 2013

1.3.1.2 **Hippocampus** - *Neuroanatomy and neurophysiology*

The hippocampus is also part of the limbic system and is known to play an important role in memory formation and decision making (Purves et al, 2012). Its name comes from the combination of the Greek words *hippos* (horse) and *kampos* (sea monster), and it was chosen based on the resemblance of the shape of the region to a sea horse. It originates from the isocortex and, because of that, it is known as a cortical-like region (similarly to the BLA; (Purves et al, 2012)). The hippocampal formation can be subdivided in different subregions: *cornu ammonis* (CA, 1–4), dentate gyrus (DG), and the subiculum (Purves et al, 2012). The CA contain three layers, with pyramidal cells as the principal excitatory cells. The dentate gyrus is morphologically distinct from CA fields and contains densely packed granule cells (neurons with relatively small cell bodies). The dentate gyrus is also one of only two regions in the brain known to house neural stem cells that are capable of differentiating into new neurons throughout adulthood (Shapiro et al., 2007; Iwai et al., 2002; Cameron and Mckay, 2001)

The hippocampus does not act as a homogeneous structure. Similar to the amygdaloid complex, subdivisions of the hippocampus are associated with different functions. For instance, the dorsal hippocampus (DH) is linked primarily to cognitive functions, while the ventral hippocampus (VH) is associated with emotional responses, such as stress and affect (Fanselow and Dong, 2010). The fear conditioning paradigm has a strong mnemonic aspect, in which the association of the CS to the US is necessary. Therefore, it is not surprising that studies in which the hippocampus was lesioned showed impairment in fear (Phillips and Ledoux, 1992; McNish et al., 1997; Gisquet-Verrier et al., 1999; Gewirtz et al., 2000; Maren and Holt, 2000; Trivedi and Coover, 2006; Zhou et al., 2016a) and anxiety (Trivedi and Coover, 2004; Raper et al., 2017) expression. However, NMDA-induced lesions of the DH one after auditory fear conditioning and ten days before re-exposure did not abolish contextual fear. In fact, the authors showed that regions of the mPFC compensated for the hippocampal loss (Zelikowsky et al., 2013). Importantly, contextual memories formed in the absence of the dorsal hippocampus were shown to fade over time, which led to the conclusion that the dorsal hippocampus although not essential in the formation of fear memory, is needed for its consolidation (Zelikowsky et al., 2012). Several studies have indicated neuronal plasticity in the DH following contextual fear conditioning (CFC), which provides more evidence for the current theory on the necessity

of the hippocampal formation for fear expression. As mentioned before, the neurocircuitry between amygdala and hippocampus has been the subject of some investigations using optogenetics. Sparta et al (2014) demonstrated that the inhibition of the BLA-entorhinal cortex pathway during CFC acquisition impaired freezing, but the inhibition during recall (or reactivation) did not have any effects. The stimulation of this same pathway was shown to enhance retention of spatial memory and impair retention of associative memory, and its inhibition resulted in trends in the opposite direction (Wahlstrom et al., 2018). The photo-stimulation of the BLA-VH pathway after CFC enhanced recall of footshock learning (Huff et al., 2016) and its inhibition had similar effects (Xu et al., 2016). Additionally, CA_1 was revealed to be activated by anxiogenic environments (Jimenez et al., 2018). The same authors showed that the optogenetic activation of the CA1-hypothalamic pathway increases anxiety and the photo-activation of the CA1-BLA pathway impaired contextual memory. In conclusion, the hippocampal formation has been revealed to have a key modulatory role in the expression of fear and anxiety, although different regions of the hippocampus are involved in distinct functions of the circuitry.

1.3.1.3 Prefrontal Cortex (PFC) - *Neuroanatomy and neurophysiology*

Early studies in which the medial PFC (mPFC) was lesioned demonstrated its importance in fear responses, especially during the extinction process (Morgan et al., 1993; Morrow et al., 1999; Quirk et al., 2000). Extinction is defined as a learned inhibition of retrieval of previously acquired memories. Electrophysiology investigations have also pointed to a role of the mPFC in fear extinction and consolidation (Milad and Quirk, 2002; Milad et al., 2004). The role of the mPFC in fear conditioning and anxiety responses has been the subject of several reviews (Davidson, 2002; Sotres-Bayon et al., 2004; Sotres-Bayon and Quirk, 2010). Later, a study revealed differential effects of two subregions of the mPFC – prelimbic (PL) and infralimbic (IF) on the expression of conditioned fear (Vidal-Gonzalez et al., 2006; de Freitas et al., 2013). The authors have shown that microstimulation of the PL resulted in increased expression of fear conditioned responses and prevented extinction, while stimulation of the IL had opposite effects. This dichotomy was further confirmed by other groups (Sierra-Mercado et al., 2011), and a pathway between the two subdivisions of the mPFC and the different nuclei of the amygdala was proposed and investigated both for fear (Sotres-Bayon et al., 2012; Gilmartin et al., 2014; Arruda-Carvalho and Clem, 2015; Giustino and Maren, 2015) and anxiety (Yamada et al., 2015).

1.3.1.4 Periaqueductal grey (PAG) - *Neuroanatomy and neurophysiology*

The periaqueductal grey (PAG) is a region dense with cell bodies that surrounds the midbrain aqueduct and it can be divided into four subcolumns: dorsomedial PAG (dmPAG), dorsolateral PAG (dlPAG), lateral PAG (lPAG), and ventrolateral PAG (vlPAG) (Bandler and Keay, 1996). The PAG is an important region in the top-down regulation of pain (Millan, 2002) and also plays a key role in the expression of fear and anxiety responses (Graeff et al., 1993; Kim et al., 1993; Watson et al., 2016). The PAG is the main effector region for the behavioural aspect of fear responses. Early studies have shown that lesions of the PAG impaired freezing expression (Liebman et al., 1970; Dostrovsky and Deakin, 1977; Watkins et al., 1983; Helmstetter and Tershner, 1994; Amorapanth et al., 1999). Likewise, its activation elicits unconditioned freezing (Siegel and Brownstein, 1975; Di Scala et al., 1987). The different subdivisions of the PAG were shown to be distinctly involved in fear conditioning. The dmPAG, dlPAG and lPAG seem to be more involved in innate responses whereas the vlPAG is involved in learned responses (Morgan et al., 1998; Watson et al., 2016; Rozeske et al., 2018). A recent study indicated that both the dmPAG and vlPAG were involved in the coding of the CS in an extinction protocol (Watson et al., 2016), suggesting that the roles of the PAG subcolumns can be more complex than thought until now. The neuronal connections of PAG with other brain regions in fear conditioning has also been examined. For instance, Rozeske et al (2018) have revealed projections from the dorsomedial PFC (dmPFC) to the lPAG and vlPAG that are selectively activated during contextual fear discrimination. Moreover, in this same study, the authors showed that optogenetic activation of this projection promoted contextual fear discrimination.

1.4 Pain and anxiety interactions

1.4.1 Anxiety and co-morbidity with pain disorders

Pain has an important emotional and affective component, and chronic pain is often associated with affective disorders, like anxiety and depression. A substantial number of studies show that patients suffering with chronic pain have higher prevalence of co-morbidity with anxiety disorders (Demyttenaere et al., 2007; Asmundson and Katz, 2009; Velly and Mohit, 2018). For example, patients with chronic pain are twice as likely to develop phobias (Pereira et al., 2017). Notably, the prevalence of depressive symptoms in

patients with chronic pain reaches 15%, against only 2.8% in patients that did not report any pain.

Moreover, there seems to be a relationship between the intensity of the pain and anxiety symptoms. People who report severe pain are more likely to have higher anxiety (Murphy et al., 2012; de Heer et al., 2014a) and post-traumatic stress disorder (PTSD) symptoms tend to be more pronounced in patients with chronic pain (Asmundson et al., 2002).

Likewise, anxiety can exacerbate painful experiences. People suffering with anxiety disorders report higher pain scores than healthy controls (Pompili et al., 2012). Additionally, improvement in anxiety symptoms resulted in a decrease of pain intensity in individuals with chronic pain (Scott et al., 2016).

1.4.2 Fear Conditioned Analgesia (FCA)

In the 1970s, three groups independently reported a phenomenon that would link pain responses to anxiety/stress exposure. Akil et al (1976) showed that the presentation of inescapable footshocks increased pain thresholds in rats. Interestingly, Mayer et al. (1975) also reported an increase in rat pain thresholds following different stressful stimuli (i.e. footshock, centrifugal rotation, and cold water). In 1977, Chance et al. (1977) paired the footshock with a neutral stimulus and observed that the presentation of the neutral stimulus triggered elevation of pain thresholds. After these observations, several studies investigated the phenomenon called stress-induced analgesia (SIA; Mayer et al., 1975; Amit and Galina, 1986; van der Kolk et al., 1989; Butler and Finn, 2009). The presentation of stressful stimuli induces robust physiological changes and results in SIA with the involvement of several neuromodulators in different brain regions (Butler and Finn, 2009).

Fear conditioned analgesia (FCA) is a subtype of SIA. In FCA, the stressful or fearful stimulus (unconditioned stimulus, US; e.g. footshock) is paired with a neutral stimulus (conditioned stimulus, CS; e.g. context) and the exposure to the previously neutral stimulus elicits pain suppression. The re-exposure of CS is enough to trigger behavioural and physiological responses similar to what is seen upon exposure to US. Thus, exposure to the CS elicits robust FCA (Finn et al., 2004; Butler et al., 2008; Butler and Finn, 2009; Rea et al., 2013), eliciting as much as 90% suppression of pain (Finn et al., 2004).

The study of FCA is important for improved understanding the physiology of endogenous analgesia and stress/fear-pain interactions, and also to facilitate discovery of novel therapeutic targets for pain disorders and their comorbidity with fear/anxiety-related disorders. A better understanding of the neurobiology of this phenomenon could potentially allow us to modulate the mechanism for therapeutic benefit. Moreover, impaired expression of SIA/FCA could point to an impairment of the descending inhibitory pathway. This information has a potential to help both the diagnostic and the choice for a future treatment of patients, which would improve success rate in pain treatments and avoid secondary morbidities such as addiction, anxiety and depression.

There are several models of FCA, but all of them involve the association of a fearful stimulus (US) with a neutral stimulus (CS) and aspect method of inducing and assessing pain. Examples of FCA models, in rodents and humans, were presented by Butler and Finn (2009). The most commonly used US is footshock that can be associated with a tone, light or the context itself. The noxious stimuli include formalin and carrageenan intraplantar injections and thermal/heat exposure.

1.4.2.1 Neurobiology of FCA

As previously described, the ascending and descending pain pathways work in an *equilibrium* and disturbances in this balance result is one of the possible origins of pathological pain. In addition, exposure to a fearful stimulus triggers endogenous analgesia, named FCA, through the activation of the descending inhibitory pain pathway. Pain and fear are mediated and modulated by complex networks which involve different neuromodulators and brain regions (see Sections 1.2 and 1.3). Some of these brain regions and their neurocircuitry are shared by both systems, particularly the PFC, amygdala, and the PAG. These sites and their internal neurophysiology are, therefore, also important in the expression and modulation of FCA.

The PFC is involved in the modulation of pain (Baulmann et al., 1999; Luongo et al., 2013; Ong et al., 2019) and fear (Sotres-Bayon and Quirk, 2010; Gilmartin et al., 2014; Wellman and Moench, 2019) responses. In rats, the medial part of the PFC (mPFC) can be further anatomically divided according to connectivity and functions in infralimbic (IL) and prelimbic (PL) subregions. Preclinical investigations have indicated that mPFC activity is

altered in pain states. Specifically, activity of both the PL and IL are reduced in acute and chronic pain (Ji et al., 2010; Ji and Neugebauer, 2014; Thompson and Neugebauer, 2018). Moreover, optogenetic activation of the PL in animals with spared nerve injury (SNI) inhibited mechanical and thermal pain responses (Lee et al., 2015). Silencing of parvalbumin positive (PV+) interneurons in the PL of SNI rats decreased tonic pain responses and mechanical and thermal sensitivity whereas the activation enhanced SNI-induced tonic pain and mechanical and thermal nociception (Zhang et al., 2015). Furthermore, several studies point to an involvement of the GABAergic (Zhang et al., 2015), glutamatergic (Kelly et al., 2016), dopaminergic (Huang et al., 2018) and cannabinoid (Kiritoshi et al., 2016; Rea et al., 2018) systems in the mPFC in the modulation of pain. As discussed before, the PL and IL have distinct roles in fear response regulation, with the IL thought to be more involved with extinction and the PL with the acquisition of fear memories (Vidal-Gonzalez et al., 2006; Herry et al., 2010b; Giustino and Maren, 2015). The importance of the PFC in FCA was shown before – MAPK signalling was attenuated in the PFC of rats expressing FCA (Butler et al., 2011). $GABA_A$ receptor antagonism in the ventral and dorso-medial hypothalamus resulted in FCA that was attenuated by microinjection of cobalt chloride (synaptic blocker) and AM251 (CB_1 antagonist) into the PL (de Freitas et al., 2013). Recently, Rea et al. (2018) have demonstrated differential roles of the endocannabinoid system in the PL and IL in FCA and expression of contextual fear in the presence of nociceptive tone.

The amygdala is a key structure in both pain processing (Neugebauer, 2015) and fear modulation (LeDoux, 2000; Myskiw et al., 2014; Adhikari et al., 2015). The amygdala is especially involved in the emotional-affective component of pain (Neugebauer, 2015). The BLA and LA receive nociceptive inputs from the thalamus, anterior cingulate cortex (ACC), and mPFC which are conveyed to the CeA. Additionally, the CeA receives direct projections with nociceptive information from the PN (Figure 1.3). The BLA and laterocapsular subdivision of the CeA responds preferentially to noxious stimulation (Neugebauer et al., 2009; Ji et al., 2010). Pain-related neuroplasticity and activity in the different subdivisions of the amygdala has been established in electrophysiological, biochemical and pharmacological studies after the induction of different pain states (Li and Neugebauer, 2004; Neugebauer, 2007, 2015; Veinante et al., 2013). The role of the amygdala in fear responses was reviewed previously (see section 1.3.1). Briefly, the inactivation or lesion of the BLA and CeA has robust effects on fear expression (see Table 1.1). The modulation of

intra-amygdalar connectivity and activity within the BLA and CeA modulates fear responses (Hartley and Phelps, 2010; Tovote et al., 2015; Izquierdo et al., 2016).

The amygdala also has an important role in the expression and modulation of FCA. The first studies investigating the role of the amygdala in FCA demonstrated that electrolytic and chemical (i.e. ibotenic acid) lesions of the BLA and CeA abolish FCA in rats (Helmstetter, 1992b; Helmstetter and Bellgowan, 1993; Watkins et al., 1993; Fox and Sorenson, 1994; Bellgowan and Helmstetter, 1996). Similarly, the microinjection of diazepam (Helmstetter, 1993) and midalozam (Westbrook, 1995) into the BLA attenuated FCA; these studies provided the first evidence for the involvement of the GABAergic system in FCA. Following these investigations, research on focused on the contribution of the opioid system. Greeley (1989) published a review in which the contribution of the opioid system to FCA is discussed. However, other non-opioid mechanisms were known to exist (Lewis et al., 1980). In 2004, Finn et al provided the first evidence for a role of the endogenous cannabinoid (endocannabinoid) system in FCA in rats (ref). In 2005, Hohmann et al. (2005) reported an endocannabinoid mechanism of SIA. The following year, these latter authors demonstrated the involvement of the endocannabinoid system in the BLA in FCA (Connell et al., 2006). Later, intra-BLA microinjections of muscimol ($GABA_A$ receptor agonist) and AM251 (CB_1 receptor antagonist/inverse agonist) were shown to prevent FCA in rats (Rea et al., 2011a, 2013b). These authors demonstrated that the endocannabinoid-mediated FCA was partially attenuated by intra-BLA administration of bicuculline ($GABA_A$ antagonist) or MPEP (2-methyl-6-(phenylethynyl) pyridine; mGluR5 antagonist) (Rea et al., 2013b). A recent study has elucidated how different cell populations are activated in the BLA and in the CeA during FCA in mice (Butler et al., 2017).

The PAG is an important region in the top-down regulation of pain (Millan, 2002) and it also plays a key role in the expression of fear and anxiety responses (Graeff et al., 1993; Kim et al., 1993; Watson et al., 2016). The antinociception caused by the activation of the descending pain pathway and the ascending transmission depends on the PAG-mediated activation of the RVM (Millan, 2002) and is modulated by different glutamatergic and GABAergic subpopulations of neurons (Samineni et al., 2017). Moreover, stimulation of the PAG resulted in robust analgesia (Mayer and Liebeskind, 1974; Walker et al., 1999). The role of the PAG in fear responses was also reviewed previously (see section 1.3.1.2). Briefly, lesions of the PAG were shown to attenuate fear in animals (Liebman et al., 1970). In humans, electrical stimulation of PAG generated reports of fear, aversion, and pain (Keene

and Figueroa, 1977). Additionally, PAG stimulation triggers behaviours that were related to anxiety and fear (Siegel and Brownstein, 1975; Schenberg and Graeff, 1978) in animals. Importantly, several studies have investigated the role of PAG in FCA expression. Lesions of the dlPAG (Kinscheck et al., 1984) and vlPAG (Bellgowan and Helmstetter, 1996) attenuated FCA. Intra-vlPAG administration of naltrexone attenuated FCA (Helmstetter and Landeira-Fernandez, 1990) and vlPAG and dlPAG blocked FCA (Helmstetter and Tershner, 1994). Intra-dlPAG microinjection of rimonabant (CB_1 receptor antagonist/inverse agonist) attenuated FCA, confirming a role of the endocannabinoid system within the PAG in FCA expression (Suplita et al., 2005; Olango et al., 2012b). Also, SIA was prevented by intra-PAG administration of CB_1 (AM251) and OX_1 (SB334867) antagonists in mice (Lee et al., 2016). Recently, chemical lesions (i.e. ibotenic acid) of vlPAG and dPAG were shown to reduce FCA in guinea pigs (Vieira-Rasteli et al., 2018).

In summary, the mPFC, the amygdala and the PAG play key roles in expression of FCA. The opioid and the endocannabinoid systems are key mediators of FCA within these regions, alongside the GABAergic and glutamatergic systems. Moreover, monoaminergic transmission within other brain regions is also involved in expression of FCA (Butler and Finn, 2009).

1.5 Peroxisome Proliferator-activated Receptors (PPARs)

A significant proportion of sections 1.5.1, 1.5.2 and 1.5.3 below has been published in Okine et al. (2018) on which I was joint first author.

1.5.1 Overview

The PPARs are ligand-dependent transcription factors that belong to the nuclear hormone superfamily of receptors. Three major isoforms have been identified: PPARα, cloned from mouse liver (Issemann and Green, 1990), PPARβ/δ, and PPARγ, both cloned from Xenopus (Dreyer et al., 1992). These three isoforms share a common structure typified by the presence of a highly conserved DNA binding domain, with two zinc finger motifs, that recognise peroxisome proliferator response element (PPRE) in the promoter regions of target genes (Desvergne and Wahli, 1999). They also contain two transcription activation domains; ligand independent AF-1 in the n-terminal domain (Delerive et al., 2002), and AF-2 in c-terminal domain, which is ligand-dependent and has a large ligand binding domain.

This large ligand binding domain makes it possible for PPARs to interact with a wide array of synthetic and natural lipid ligands.

The PPAR signalling system comprises the three isoforms of PPARs – PPARα, PPARβ/δ and PPARγ – and their endogenous ligands (mainly, but restricted to, N-acylethanolamides – NAEs – such as palmitoylethanolamide – PEA, oleylethanolamide, OEA, and anandamide, AEA) together with the biological mechanisms for the synthesis and metabolism of these ligands (Table 1.2).

Table 1.2: Endogenous ligands at PPARs.

Endogenous Ligands	*PPARα*	*PPARβ/δ*	*PPARγ*	*Reference*
OEA	✓		✓	(Fu et al., 2003a; O'Sullivan et al., 2006)
PEA	✓	✓	✓	(LoVerme et al., 2005; Paterniti et al., 2013)
AEA	✓		✓	(Bouaboula et al., 2005; Sun et al., 2007)
2-AG metabolites	✓		✓	(Kozak et al., 2002; Rockwell et al., 2006; Kaczocha et al., 2014)
Oleamine	✓		✓	(Fakhfouri et al., 2012; Granja et al., 2012)
Virodhamine	✓			(Sun et al., 2007)
Noladin ether	✓			(Sun et al., 2007)
N-arachidonoyl-dopamine (NADA)			✓	(O'Sullivan et al., 2009)
Unsaturated fatty acids	✓	✓	✓	(Forman et al., 1997; Kliewer et al., 1997; Waku et al., 2009)
Saturated fatty acids	✓	✓	✓	(Kliewer et al., 1997; Waku et al., 2009)
Palmitic acid	✓			(Aoyama et al., 2002)
Palmitoleic acid	✓			(Chimin and Torres-Leal, 2013)
Oleic acid	✓			(Ziamajidi et al., 2013; Alen et al., 2018)
Linoleic acid	✓		✓	(Moya-Camarena et al., 1999; Bull et al., 2003; Schopfer et al., 2005)
Arachidonic acid (and metabolites – HETE)	✓			(Caijo et al., 2005; Trombetta et al., 2007)
Eicosapentaenoic	✓	✓		(Forman et al., 1997; Xu et al., 1999)
Serotonin metabolites			✓	(Waku et al., 2010b)
Phytanic acid	✓			(Heim and Johnson, 2002)
Carbaprostacyclin ($cPGI_2$)		✓		(Kurtz et al., 2010)
3-hydroxy-(2,2)-dimethyl butyrate	✓			(Chakrabarti et al., 2019)

1.5.2 Mechanism of action

PPARs exist as heterodimers with the retinoid X receptor (RXR), bound to co-repressor proteins in the inactive state. Upon ligand activation, the co-repressors dissociate from the PPAR/RXR complex, allowing for the recruitment of co-activators. The activated PPAR/RXR-co-activator complex subsequently binds to specific DNA sequences or PPRE, resulting in the transcriptional activation of target genes (Green et al., 1992, Tugwood et al., 1992). Genes regulated by PPARs via this PPRE-dependent mechanism are mainly involved in lipid and lipoprotein metabolism (Tugwood et al., 1992). Alternative non-genomic mechanisms of action have been reported, especially for PPARα which has known anti-inflammatory effects (Delerive et al., 2001). These latter mechanisms involve inhibition of NF-kB and AP-1 inflammatory signalling and the consequent trans-repression of pro-inflammatory genes such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and tumour necrosis factor-alpha (TNF-α) (Crisafulli and Cuzzocrea, 2009, Cuzzocrea et al., 2008, Delerive et al., 2000). These anti-inflammatory consequences of PPARα activation are fundamental to the role of this receptor in modulating both inflammatory and neuropathic pain.

1.5.3 PPARs and pain

1.5.3.1 Expression of PPARs in key components of the pain pathway

A role for PPAR signalling in pain processing is suggested by studies demonstrating the presence of the different PPAR isoforms at key peripheral, spinal and supraspinal sites involved in pain processing (Table 1.3). Within the periphery, PPARα is expressed in the dorsal root ganglion (DRG) (LoVerme et al., 2006). Unlike PPARα, to my knowledge, the expression of PPARβ/δ in the DRG remains unexplored. Despite the paucity of data on the distribution pattern of PPARα on nociceptive primary afferents (Aδ-fibres and C-fibres), the reported analgesic effects of PPARα agonists administered locally/peripherally in animal models of inflammatory and neuropathic pain suggest a modulatory influence on peripheral nociceptive afferents such that activation of PPARα in the DRG results in the suppression/silencing of nociceptive afferent fibre firing. However, the validation of this hypothesis requires further characterisation of PPARα in DRG nuclei using double-labelling IHC or *in situ* hybridisation techniques to elucidate the neuronal subtypes in which these receptors are expressed.

PPARα expression in the spinal cord has also been demonstrated in previous studies (Benani et al., 2004, Okine et al., 2015). The functional relevance of PPARα signalling in the spinal cord to nociceptive processing is demonstrated, at least in part, by the reported increases in PPARα activation or expression in animal models of chronic inflammatory and neuropathic pain states. For example, using electrophoretic mobility-shift assay (EMSA), Benani and colleagues were able to demonstrate a rapid increase in activation of the PPARα isoform in the rat spinal cord after CFA injection into the hind paw (Benani et al., 2004). Moreover, increased PPARα expression in the ipsilateral spinal cord was observed in the rat spinal nerve ligation model of neuropathic pain (Okine et al., 2015). Furthermore, it has been shown that down-regulation of PPARα in the spinal cord contributes to augmented peripheral inflammation and inflammatory hyperalgesia in diet-induced obese rats (Wang et al., 2014). While the pathophysiological relevance of PPARα activation or changes in PPARα expression in the spinal cord during hyperalgesia requires further investigation, these findings provide evidence for PPARα as a potentially important player in spinal pain processing. In addition to PPARα, both PPARβ/δ and PPARγ are also expressed in the spinal cord. Increased PPARγ expression in the spinal trigeminal caudalis 3 weeks after trigeminal inflammatory compression injury in mice has been reported to play a significant role in trigeminal nociceptive transmission, as demonstrated by the attenuation of whisker pad mechanical allodynia (Lyons et al., 2017a), and identifies PPARγ as a potential therapeutic target for orofacial neuropathic pain.

In comparison, there is a paucity of data on the expression or activation of PPARβ/δ in the spinal cord in inflammatory or neuropathic pain states. In this regard, further characterisation of PPARβ/δ is essential to establish whether such changes in expression or endogenous activation are apparent, and the extent to which they may contribute to spinal pain processing.

All three PPAR isoforms are widely expressed supraspinally (Table 1.3), in key brain regions involved in pain processing. The expression of PPARs at key relay sites such as the thalamus and the midbrain periaqueductal grey (PAG) may reflect a role for PPAR signalling in modulating the activity of ascending and descending pain pathways. Furthermore, the presence of PPARs within cortical regions and the amygdala suggests potential involvement of PPAR signalling in modulating cognitive or affective components of pain. Whilst there is currently no direct evidence in support of this hypothesis, this view is however consistent with the role of both the cortex and amygdala as key brain regions

involved in the modulation of the cognitive-affective components of pain. For reviews, see (Fuchs et al., 2014, Neugebauer, 2015).

Table 1.3: Expression of mRNA or protein for PPAR isoforms within neuroanatomical loci involved in pain and fear/anxiety (from Okine et al. 2018).

Brain Region	PPAR-α expression	PPAR-β/δ expression	PPAR-γ expression
Frontal Cortex	✔[1]	✔[1]	✔[1]
Pre-frontal Cortex (PFC)	✔[2]	✔[2]	✔[2]
Hippocampus	✔[1]	✔[1]	✔[1]
Thalamus	✔[1]	✔[1]	✔[1]
Hypothalamus	✘[1]	✔[1]	✔[1]
Basal Ganglia	✔[1, 2]	✔[1, 2]	✔[1, 2]
Amygdala	✔[2]	✔[2]	✔[2]
PAG	✔[3]	✔[3]	✔[3]
Rostroventral Medulla (RVM)	?	?	?
Ventral Tegmental Area (VTA)	✔[2]	✔[2]	✔[2]
Spinal Cord	✔[*1]	✔[1]	✔[*1]
Astrocytes	✔[1]	✔[1]	✔[1]
Oligodendrocytes	✘[1]	✔[1]	✘[1]

**Not expressed in all laminae; [?]Expression not known to date [1]According to Moreno et al, 2004; [2]According to Wander et al, 2016; [3]According to Okine et al, 2016.2*

1.5.3.2 Evidence from pharmacological or genetic manipulation studies for a role of PPARs in pain

Pharmacological or genetic manipulation of PPARα and PPARγ using selective agonists, antagonists or gene knockout approaches specifically targeting these receptors within the pain pathways has been shown to alter nociceptive processing, demonstrated by changes in electrophysiological recordings of neuronal activity or behavioural responses in animal models of inflammatory and neuropathic pain (Figure 1.5; see Appendix A for a table summarising studies that investigate PPAR signalling and pain). Both PPARα and PPARγ regulate the release of pro-inflammatory mediators associated with tissue or nerve injury through the inhibition of pro-inflammatory signalling pathways such as NF-κB activation (Cuzzocrea et al., 2008, Delerive et al., 2000) and suppression of downstream pro-inflammatory molecules including COX-2 and iNOS (D'Agostino et al., 2009), two key

players in the development of chronic pain states. Most pharmacological studies to date demonstrate antinociceptive effects of both endogenous and synthetic agonists of PPARα and PPARγ in animal models of inflammatory and neuropathic pain (Okine et al, 2018). It is pertinent to note that a significant proportion of preclinical studies investigating the role of endogenous PPAR ligands in nociceptive processing have mainly focused on the effects of PEA in the peripheral and central nervous systems, with relatively little attention given to the role of OEA. One possible reason that may account for this apparent bias is the reported activation of the transient receptor potential cation channel subfamily V member 1 (TRPV1; the vanilloid receptor), a pro-nociceptive non-selective cation channel, by OEA (Ahern, 2003). Thus, it is possible that any PPAR-mediated analgesic effects of OEA are likely to be nullified by its TRPV1-mediated pro-nociceptive effects, as previously demonstrated in an animal model of neuropathic pain (Guida et al., 2015).

The pharmacological effects of PEA involve both transcription-dependent and transcription-independent or non-genomic mechanisms. While the former account primarily for the anti-inflammatory effects associated with PPAR activation, the non-genomic mechanisms are thought to underlie the rapid antinociceptive effects of not only PEA, but also other synthetic PPAR agonists in animal models of inflammatory and neuropathic pain (Churi et al., 2008, LoVerme et al., 2006). It is, however, important to note that the non-genomic mechanisms mediating the effects of PEA are not independent of PPAR expression or activation. Indeed, evidence from studies with PPAR knockout mice suggests that the modulation of medium and large Ca^{2+} channels associated with the rapid antinociceptive effects of PEA and other synthetic PPARα agonists on inflammatory pain behaviour in mice are contingent upon PPARα receptor expression in the DRG (LoVerme et al., 2006). Given that these rapid antinociceptive effects are incompatible with the duration of longer-term transcription-dependent mechanisms, the modulation of Ca^{2+} channels in this instance may be a by-product of protein-protein interactions induced by changes in PPAR protein conformation following the binding of agonist to the receptor. The non-genomic effects of PEA may also involve the indirect activation of other receptor signalling systems such as the cannabinoid$_1$ (CB_1) receptor, mediated by AEA. In this regard, competition for fatty acid amide hydrolase (FAAH)-mediated hydrolysis, by PEA, is thought to provide a 'sparing effect' on AEA hydrolysis by FAAH, resulting in enhanced signalling at endocannabinoid targets, in particular CB_1 or CB_2 receptors, to produce analgesia. A role for CB_1 receptors in the antinociceptive effects of PEA injected directly into the anterior cingulate cortex (ACC) in

the rat formalin test has recently been demonstrated (Okine et al., 2016b). Moreover, given the preferential binding of AEA over PEA to PPARγ (Bouaboula et al., 2005), it is possible that entourage-mediated signalling involving AEA likely underpins the PPARγ-mediated antinociceptive effects of PEA (Costa et al., 2008). Indeed, AEA binds to and activates PPARα in addition to PPARγ (Bouaboula et al., 2005). The analgesic effects of PPAR agonists may also be mediated via modulation of cellular organelles. For example, a combination drug therapy of the synthetic PPARγ agonist pioglitazone with D-cycloserine attenuates chronic orofacial neuropathic pain and associated anxiety by improving mitochondrial function following trigeminal nerve injury (Lyons et al., 2017b). Furthermore, given the involvement of both genomic and non-genomic mechanisms in mediating the effects of PPAR agonists, future studies aimed at determining which mechanisms are predominant in different types of pain will be important for the optimisation of the analgesic effects of PPAR agonists.

It is however important to note that while the weight of evidence is in favour of antinociceptive effects of PPARα or PPARγ activation at multiple sites within the pain pathway, recent findings also reveal a pain permissive or facilitatory role for PPAR signalling in discrete brain regions such as the ACC (Okine et al., 2016a, Okine et al., 2014). Intra-ACC injection of GW6471 (selective PPARα antagonist) or GW9662 (selective PPARγ antagonist) significantly suppressed the onset of formalin-evoked nociceptive behaviour in rats (Okine et al., 2016a). Such permissive or facilitatory roles of endogenous PPAR activation within the ACC may allow the animal to perceive pain and take the necessary actions to escape from immediate danger.

The specific role of PPARβ/δ activation in pain processing remains largely unknown, despite molecular evidence demonstrating the presence of the receptor at key sites within the pain pathway such as the spinal cord, thalamus and PAG. However, in a previous study, administration of GW0742, a selective PPARβ/δ receptor agonist (0.1mg/kg/i.p. for 4 days) significantly decreased mechanical and thermal hyperalgesia in adult male Wistar rats, induced by carrageenan injection into the hind paw compared with vehicle-treated controls. These effects were reversed in the presence of the selective PPAR β/δ antagonist GSK0660 (0.3mg/kg/i.p. for 4 days) (Gill et al., 2013). These findings demonstrate the potential of PPAR β/δ agonists as therapeutic agents for the treatment pain. Further preclinical studies are however needed to understand fully the extent to which PPARβ/δ-mediated signalling modulates nociceptive transmission within the CNS.

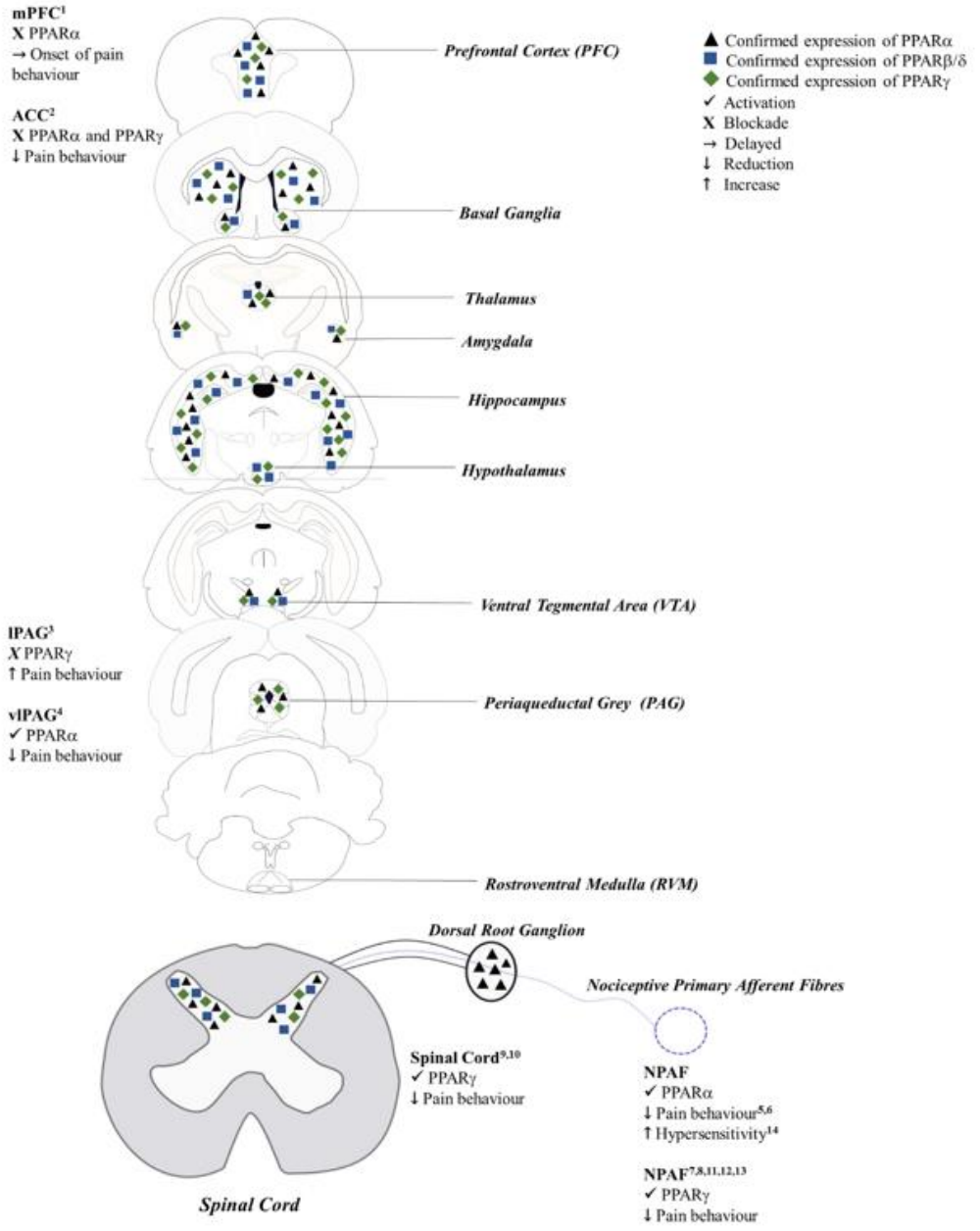


Figure 1.5: Anatomical localization of PPAR isoforms in key components of the pain pathway and their role in pain modulation (from *Okine et al. 2018*).

(1) Okine et al. (2014); (2) Okine et al. (2017); (3) Okine et al. (2016); (4) de Novellis et al. (2012); (5) LoVerme et al. (2006); (6) Russo et al. (2007); (7) Hasegawa-Moriyama et al. (2013); (8) Mansouri et al. (2017a,b); (9) Churi et al. (2008); (10) Griggs et al. (2015); (11) Saito et al. (2015); (12) Hasegawa-Moriyama et al. (2012); (13) Takahashi et al. (2011); (14) Sagar et al. (2008); (15) D'Agostino et al. (2009); (16) Moreno et al. (2004); (17) Warden et al. (2016); (18) Churi et al. (2008); (19) Maeda et al. (2008); (20) Chakravarthy et al. (2007).

1.5.3.3 Evidence from clinical trials

Over the last couple of decades, the analgesic effects of PEA, an endogenous PPAR agonist, or its derivatives, have been demonstrated in multiple clinical trials in different pain conditions. In a recent comprehensive review of 21 clinical trials, Gabrielsson *et al.*, reported that oral or sublingual treatment with PEA or micronized PEA (PEA-μm; reduced crystal particles of PEA that enhance the dissolution and reduce the absorption variability) reduced pain intensity in patients with neuropathic and inflammatory joint pain phenotypes (Gabrielsson *et al, 2016*). These treatments were not associated with significant side effects. Similar reports of the analgesic effects of PEA in clinical trials have been discussed in another comprehensive review by Hesselink and Hekker (2012). These studies report that administration of PEA (doses ranging from 300 to 600 mg/day; mostly orally administrated as tablets) is effective against a range of pain conditions including neuropathic pain, low back pain and postoperative pain.

In contrast, Andresen and colleagues report that a 12-week treatment with PEA-μm did not alleviate pain in patients with spinal cord injury-induced neuropathic pain, compared to placebo-treated patients (Andresen et al., 2016). The authors however point out that the limited knowledge on PEA-μm pharmacokinetics, including information on diffusion to the cerebrospinal fluid, make it difficult to draw more specific conclusions. It is also possible that the heterogeneity in the population of spinal cord injury pain phenotypes could have impacted on the outcome of this study. These clinical effects of PEA however, while suggestive of a role for PPAR signalling, do not necessarily rule out the involvement of other receptor systems, given the multiple signalling pathways mediating the pharmacological effects of PEA as demonstrated in preclinical studies. In this regard, the use of synthetic PPAR agonists in clinical trials may be more beneficial and informative. In keeping with this line of argument, a more defined role for PPAR signalling in modulating human pain conditions was demonstrated in a study by Smith and colleagues, who reported a reduction in occurrence of myalgia, a muscle-skeletal pain disorder, in men receiving clofibrate, an approved PPARα agonist used clinically for the treatment of dyslipidemia (Smith et al., 1970). However, subsequent attempts at replicating these early promising results using other fibrates to alleviate muscular pain have not been successful (Biga et al., 2005). Nevertheless, these drugs were found to be effective in attenuating pain associated with rheumatoid arthritis and osteoarthritic pain (van Eekeren et al., 2013). These findings

indicate that that synthetic PPARα agonists can have analgesic effects in specific types of pain.

Synthetic agonists of PPARγ are currently used clinically as insulin sensitizers in the treatment of non-insulin dependent (type two) diabetes. However, despite preclinical evidence demonstrating their analgesic effects in a variety of animal models of inflammatory and neuropathic pain, to my knowledge there are currently no published clinical studies investigating their effects on pain in human subjects or patients. Similarly, there is a paucity of clinical studies investigating the effects of synthetic PPARβ/δ agonists on pain.

1.5.3.4 A potential role for PPAR signalling in interactions between pain and negative affect

The close relationship between stress (and stress-related disorders such as anxiety and depression) and chronic pain is now widely recognised (Jennings et al., 2014, Olango and Finn, 2014). Although, the role of PPAR signalling in the modulation of stress-pain interactions remains largely unexplored, the abundant expression of PPARs in key brain regions such as the amygdala and PAG, and the availability of endogenous PPAR ligands in these brain structures, supports a potential role for the PPAR signalling system in stress–pain interactions and as a potential therapeutic target for the treatment of comorbid chronic pain and affective disorders. This view is also consistent with recent demonstrations of enhanced second phase formalin-evoked nociceptive behaviour following selective blockade of PPARγ in the lateral PAG in Wistar-Kyoto (WKY) but not Sprague-Dawley (SD) rats (Okine et al., 2016a). The WKY rat strain is stress-hyperresponsive and exhibits a hyperalgesic phenotype to nociceptive stimuli compared with SD rats, and is considered a suitable genetic model for studying stress-pain interactions (Burke et al., 2010, Rea et al., 2014, O' Mahony et al., 2013). While the specific contribution of PPARγ signalling to the stress hyperresponsive phenotype of WKY rats is not currently clear, the differential effects of pharmacological modulation of PPARγ in the lateral PAG on formalin-evoked nociceptive behaviour in SD and WKY rats suggests an important role for this receptor in a genetic background that is prone to stress and hypersensitivity to nociceptive stimuli. These findings also suggest that PPARγ-mediated signalling in the lateral PAG may represent a potential therapeutic target for future development of effective therapies for treating comorbid chronic pain and stress-related disorders such as anxiety and depression. The

therapeutic potential of PPARγ for treatment of pain and mood disorder comorbidity is also supported by evidence that pioglitazone attenuates CCI-induced depression-related behaviour in the forced swim test in rats (Garg et al., 2017)), reduces anxiety-like behaviour in a mouse model of chronic orofacial neuropathic pain (Lyons et al., 2017b) and augments both the anti-depressant and the antinociceptive effects of fluoxetine in the rat CCI model of neuropathic pain (Murad and Ayuob, 2015). Additional studies on the therapeutic potential of PPAR agonists (including those for PPARα and PPARβ/δ) for treatment of the affective/emotional component of chronic pain are warranted.

1.5.4 PPARs and fear/anxiety

The three subtypes of PPARs are expressed in brain regions associated with anxiety (Moreno et al., 2004; Warden et al., 2016), namely the amygdala, PFC, PAG, and hippocampus. Levels of endogenous ligands at PPARs have also been shown to be increased in response to stress or anxiety (Bluett et al., 2014; Hillard, 2018) and the enzyme FAAH was linked to anxiogenic effects (Burman et al., 2016) and to structural modifications in the BLA following chronic stress (Hill et al., 2013). A recent clinical study has also shown that the levels of OEA in the blood are significantly lower in PTSD patients compared to control subjects (Wilker et al., 2016). A strong trend in the same direction was also observed for PEA. Additionally, administration of PEA attenuated aggressiveness in a social isolation model for PTSD in mice (Locci et al., 2017). Both OEA and PEA are endogenous ligands at PPARs and may point towards an important role of this system in anxiety-related disorders in humans. Despite these indications, very little research has investigated the role of PPARs in anxiety.

In 2009, Fernandez et al. (2009) revealed that naringin, a bioflavonoid isolated from citrus fruits, had anxiolytic and antidepressant effects. However, only recently naringin was found to be a natural ligand (derived directly from citric fruits) at PPARγ (Mani and Sadiq, 2014). Another study indicated that male seipin (integral membrane protein) knockout (Seipin-KO) mice displayed anxiety- and depression-like behaviours, which were associated with decreased levels of PPARγ mRNA and protein in the hippocampus and cortex (Zhou et al., 2014). Importantly, the administration of rosiglitazone, an agonist at PPARγ, attenuated the anxiogenic profile of male Seipin-KO mice. Although these two studies were pointing towards a role for PPARγ in the modulation of anxiety responses, none of them directly manipulated PPARγ signalling, expression or activity. In 2016, a very interesting study

addressed this issue. It revealed that PPARγ genetic deletion had anxiogenic effects in mice (Domi et al., 2016). In this same investigation, the authors showed that systemic and intra-amygdalar injections of pioglitazone (PPARγ agonist) reduced stress-induced anxiety behaviour in rats, and that these effects were blocked by the administration of the PPARγ antagonist GW9662. Importantly, the systemic administration of GW9662 alone did not alter anxiety-related behaviour. Furthermore, rosiglitazone was shown to elicit antidepressant-like (increased latency to immobility in the forced swim test) and anxiolytic (more time spent in the open arm in the elevated plus maze) behavioural effects in wild-type mice and pre-treatment with the selective PPARγ antagonist GW9662 blocked the effects of rosiglitazone. (Guo et al., 2017). Recently, administration of pioglitazone was shown to attenuate harmaline-induced anxiety-like (through activation of olivary neurons; Hilbert et al, 2005) behaviours and spatial learning and memory impairments (Aghaei et al., 2019), similar to what was observed with rosiglitazone-treated animals. Likewise, Youssef et al. (2019) have shown that the administration of GW9662 blocked the anxiolytic effect of beta-caryophyllene (via agonism at CB_2 receptors) in rats. A different study demonstrated that repeated stress decreased protein PPARγ expression in the amygdala, and treatment with anxiolytics recovered PPARγ expression (Liu et al., 2018). The role of PPARγ in fear responses has also been investigated. Gemma et al. (2004) demonstrated that young and aged rats fed with a diet rich in rosiglitazone had increased freezing duration in a context-induced fear protocol. In addition, the levels of PEA where shown to be increased in the BLA of fear-conditioned rats (Rea et al., 2013c).

The role of PPARα in anxiety is under studied. Recently, it has been reported that the anxiolytic-like effects of URB597 (FAAH inhibitor) were not reversed by the PPARα antagonist GW6471 (Danandeh et al., 2018). Similarly, systemic administration of the PPARα antagonist MK886 did not alter anxiety-like behaviour in the open field test (Panlilio et al., 2009). These two studies suggest that PPARα, contrary to what has been observed for PPARγ, is not involved in the modulation of anxiety responses. A possible role of PPARβ/δ in anxiety modulation or mediation is still unexplored.

1.5.5 PPARs and cognition

All three subtypes of PPARs are expressed in important regions associated with cognition, stress and emotional responses (Moreno et al., 2004; Warden et al., 2016) such as the basal

ganglia, amygdala, PFC and thalamus, with lower expression in the hippocampus. Additionally, administration of endogenous ligands at PPARs, or manipulation of their levels, has been shown to enhance cognitive performance (Campolongo et al., 2009a; Goonawardena et al., 2011; Morena et al., 2014; Kramar et al., 2017; Rueda-Orozco et al., 2017; Scuderi et al., 2018; Segev et al., 2018; Zimmermann et al., 2018; Boccella et al., 2019). However, there are very few direct investigations of the role of PPARs in cognition. A number of studies have investigated the effects of FAAH inhibitors on mnemonic tasks, but the results are often associated with CB_1 receptor modulation. Nevertheless, a few studies indicate a possible modulatory effect of PPARs in memory and learning processes, in subjects with preserved mnemonic abilities. Mazzola et al (2009) have shown that the administration of URB597 before the learning trial of a passive avoidance test enhanced the learning of the task. Moreover, this enhancement was attenuated by the administration of the PPARα antagonist, MK886. Following this result, these authors also demonstrated that the administration of a PPARα agonist, WY14643, produced learning enhancement effects similar to those observed with URB597, effects also blocked by MK886. A study from Campolongo et al. (2009) indicated that the administration of OEA improved learning of passive avoidance and spatial memory tasks when given immediately post-training and that the actions of OEA were mimicked by the PPARα agonist GW7647 and are absent in PPARα null mice. Recently, Ratano et al. (2017) showed that the cognitive enhancing effects of URB597 were dependent on PPARα, as well as CB_1 receptors and $TRPV_1$. Additionally, pioglitazone administration improved short-term mnemonic performance in wild type mice, but the authors did not examine if this effect was mediated by PPARγ (Masciopinto et al., 2012). Together, these studies provide evidence for a modulatory role of the PPAR signalling system in memory acquisition and consolidation. Recently, PPARα knockout (PPARα-KO) mice showed enhanced fear learning compared to WT counterparts (Chikahisa et al., 2019). Interestingly, in this same study, the authors found that PPARα-KO mice had increased levels of dopamine in the amygdala, and the administration of a D1 antagonist attenuated the increased fear learning observed in KO animals.The studies mentioned above used animals with intact mnemonic abilities in order to investigate the role of PPARs in cognition. However, the majority of the research on PPARs and memory has been carried out in animal models of Alzheimer's disease (AD), in which the subject has impaired mnemonic abilities, mimicking the symptoms of this neurodegenerative disorder. Because there are a significant number of studies demonstrating neuroprotective anti-

inflammatory effects of PPARs, especially PPARγ, they became candidates for therapeutic interventions for AD patients. Therefore, a few studies are dedicated to the examination of the effects of PPAR-based approaches for the treatment of the cognitive decline associated with AD, at both preclinical and clinical levels. For instance, chronic treatment with rosiglitazone (PPARγ agonist) reduced spatial (Pedersen et al., 2006; Escribano et al., 2010; Toledo and Inestrosa, 2010; O'Reilly and Lynch, 2012; Song et al., 2014; Qian et al., 2016; Zhou et al., 2016b), recognition (Escribano et al., 2010), and associative (Rodriguez-Rivera et al., 2011) memory loss in different mice transgenic models of AD. Likewise, treatment with rosiglitazone improved contextual associative learning and the co-administration with a PPARγ antagonist prevented this enhancement (Denner et al., 2012). Importantly, both Denner et al. (2012) and O'Reilly and Lynch (2012) demonstrated that rosiglitazone treatment to wild type mice does not affect cognitive functions. In a follow-up study, it was shown that the cognitive enhancing effects of PPARγ agonism are associated with a normalisation of the I-O relationship of EPSCs in the dentate gyrus which were shown to be altered (lower in amplitude and higher in frequency) in an AD mouse transgenic model (Nenov et al., 2014). Rosiglitazone (Xu et al., 2014) and telmisartan (Shindo et al., 2012) improved spatial memory in a Aβ42 oligomer-induced memory impairment model. Recently, a combined treatment of leptin and pioglitazone also resulted in enhanced spatial memory in a transgenic mouse model of AD (Fernandez-Martos et al., 2017). Pioglitazone and a pan-agonist of PPARs (GFT1803) both partially reverted cognitive deficit of the APP/PSI transgenic mouse model of AD (Kummer et al., 2015) and nanoparticles of pioglitazone attenuated cognitive deficits in this same model (Silva-Abreu et al., 2018). Pioglitazone also improved memory functions in a scopolamine-induced (Allami et al., 2011; Almasi-Nasrabadi et al., 2012, 2014; Gupta and Gupta, 2012), streptozotocin-induced (Pathan et al., 2006; Kaur et al., 2009; Ponce-Lopez et al., 2011; Prakash et al., 2015), LPS-induced (Ekladious and El Sayed, 2019), and other transgenic (Nicolakakis et al., 2011; Masciopinto et al., 2012) models of AD cognitive deficits. Likewise, pre-treatment with BADGE, a PPARγ antagonist, abolished the beneficial effect of lisinopril/telmisartan combined treatment on spatial memory in a streptozotocin-induced AD memory impairment (Singh et al., 2013).

Clinical studies also revealed a potential positive effect of PPARγ agonists on the treatment of cognitive deficits in AD patients. In a preliminary study, Watson et al. (2005) indicated that treatment with rosiglitazone may offer an alternative for the treatment of cognitive

decline associated with AD. In a more extensive study, treatment with rosiglitazone (8mg/kg) resulted in improvements in attention and memory retention, but only in patients that did not have an ApoE4 allele (Risner et al., 2006). These findings conflict with a phase III clinical study which showed that treatment with rosiglitazone did not have an effect on cognitive function (Gold et al., 2010). Other clinical studies have also examined possible cognitive enhancement effects of treatment with pioglitazone in the cognitive decline of AD patients. Hanyu et al. (2009) and Sato et al. (2011) indicated that treatment with pioglitazone improved cognition in AD patients with type II diabetes. More clinical studies are needed to elucidate the beneficial effect of PPARγ antagonist in cognitive deficits and to develop new strategies for their use.

PPARα and PPARβ/δ has also been investigated in relation to potential effects on cognitive performance in AD models. Chronic administration of PEA reduced (low dose) or prevented (high dose) cognitive performance impairments induced by intracerebral injection of amyloid-β-25-35, a model of AD in mice. These effects were absent in PPARα null mice and were mirrored by chronic administration of the PPARα agonist GW7647 (D'Agostino et al., 2012). Similar to pioglitazone, the systemic administration of the PPARα agonist WY14643 enhanced mnemonic performance in scopolamine-induced memory deficits (Xu et al., 2016b). In addition, administration of the PPARβ/δ agonist GW0742 significantly attenuated the cognitive impairment induced by the intranigral injection of 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) (Das et al., 2014).

PPARs also modulate cognitive deficits in other models of memory impairment. In particular, agonists of PPARγ were shown to reverse mnemonic deficits in several models. For instance, pioglitazone improved spatial memory and/or passive avoidance performance in a morphine-induced (Babaei et al., 2012), harmaline-induced (Aghaei et al., 2019), and in insulin-resistance related (Gad et al., 2016) mnemonic impairment. Additionally, the pre-treatment of pioglitazone and fenofibrate (PPARα agonist) protected against MPTP-induced mnemonic deficits (Barbiero et al., 2014). Rosiglitazone (Fei et al., 2015; Kariharan et al., 2015; Ma et al., 2015; Patel et al., 2016) and pioglitazone (Jiang et al., 2012; Yin et al., 2013; Jain et al., 2016) also ameliorated memory function in diabetes-induced mnemonic impairment in rats. The dietary inclusion of rosiglitazone also improved associative memory in aged and young rats, compared to aged animals receiving standard diet (Gemma et al., 2004). Administration of telmisartan, a PPARγ partial agonist, also ameliorated spatial

memory in ischemia models (Haraguchi et al., 2010; Washida et al., 2010; Gao et al., 2018). This effect was blocked by the administration of a PPARγ antagonist, GW9662. Interestingly, dietary administration of a PPARβ agonist (GW0742) did not prevent hippocampal-dependent cognitive impairment after whole brain irradiation (Schnegg et al., 2013) and rosiglitazone treatment after traumatic brain injury did not improve mnemonic functions (Liu et al., 2016). One clinical investigation explored the possibility of pioglitazone as an alternative treatment for multiple sclerosis (MS) (Pershadsingh et al., 2004). The authors described one patient who received an oral treatment of pioglitazone for one month and showed an improvement in cognitive functions and other symptoms of MS.

Other lines of work have also provided evidence for associations between memory enhancement and PPAR signalling. For example, polyunsaturated fatty acid diets with low n-6: n-3 ratios resulted in improved spatial learning and memory, effects correlated with an up-regulation of PPARα and PPARγ in the hippocampus (Hajjar et al., 2012). Likewise, the ablation of FABP5 - a fatty acid-binding protein that was shown to shuttle arachidonic acid to the nucleus, thus activating PPARβ/δ - reduced PPARβ/δ activity which caused a significant impairment of hippocampus-based memory (Yu et al., 2014). Another study demonstrated that sevoflurane-induced neurotoxicity and learning and memory impairment was ameliorated by down-regulation of miR-27a-3p (non-coding RNA that functions as a tumour suppressor), and this effect was mediated through the PPAR-γ signalling pathway (Lv et al., 2017).

In summary, only a few studies have investigated a possible role of PPARs in memory and learning in animals with intact mnemonic function. These studies have demonstrated that pharmacological and genetic manipulation of PPARα affects acquisition (Mazzola et al., 2009b) and consolidation (Campolongo et al., 2009) of memories, thus indicating a modulatory role for these receptors in cognition. Moreover, a significant body of preclinical and clinical research has shown positive effects of PPAR activation, particularly PPARγ, in subjects with impaired mnemonic functions. These effects are most likely related to the well-known anti-inflammatory and neuroprotective properties of PPARs, but the possibility of a direct effect on memory formation and consolidation cannot be disregarded.

1.6 Hypothesis and aims of thesis

The studies discussed above suggest that PPARα and PPARγ play important roles in pain processing as well as in anxiety and fear responses, while the involvement of PPARβ/δ in pain, fear and anxiety still needs to be investigated. However, a possible role of PPARs in fear/anxiety-pain interactions remains unexplored. Moreover, the role of PPARs in the amygdala, a key region for both nociception and fear processing, on conditioned fear and acute inflammatory pain is still unknown. Finally, only a few studies have investigated the involvement of PPARs in cognitive processes in animals with preserved mnemonic abilities, while no studies have investigated PPAR regulation of cognition in the presence of pain, and so these questions require further study.

I hypothesise that the blockade of PPAR signalling (1) increases inflammatory pain responses, (2) attenuates FCA, (3) increases anxiety-like responses, and (4) impairs cognitive processing; in addition, I propose that this modulation is mediated by alterations in NAE and neurotransmitter levels in two key regions involved in pain and fear processing – amygdala and hippocampus. Furthermore, I theorise that PPAR signalling within the BLA and the CeA is involved in conditioned fear, nociception and FCA, through modulation of NAEs and neurotransmission in the BLA and CeA. Finally, given previous reports on the role of PPARs in anxiety and cognition, I hypothesise that PPAR signalling blockade is anxiogenic and impairs mnemonic processing.

The overarching aim of this thesis is to advance understanding of the role of PPARα, PPARβ/δ and PPARγ in acute and chronic inflammatory pain, conditioned fear, FCA, innate anxiety and cognition in rats. An additional aim is to investigate the influence of pain on PPAR-mediated regulation of conditioned fear responses, innate anxiety and cognition.

Therefore, chapter 2 explores the effects of systemic administration of PPARα, PPARβ/δ and PPARγ antagonists on formalin-induced inflammatory pain, conditioned fear and FCA and examined associated changes in the levels of neurotransmitters, endocannabinoids, and NAEs in the amygdala and hippocampus. The third chapter examines the effects of the blockade of PPARs in the BLA on formalin-induced inflammatory pain and FCA, and on conditioned fear in the presence and absence of a nociceptive tone; associated alterations in neurotransmitter, endocannabinoid and NAE levels were also examined. Similarly, the fourth chapter investigates the effects of the blockade of PPARs expressed in the CeA on formalin-induced inflammatory pain and FCA, and on conditioned

fear in the presence and absence of a nociceptive tone; again, associated changes in neurotransmitter, endocannabinoid and NAE levels were examined. The fifth chapter focuses on the investigation of the effects of systemic administration of PPARα, PPARβ/δ and PPARγ antagonist and PEA (agonist for PPARs) on anxiety and cognition in the presence versus absence of chronic inflammatory pain induced by complete Freund's adjuvant.

Chapter 2: Effects of systemic administration of PPAR antagonists on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats

2.1 Introduction

PPARs are ligand-dependent transcription factors and part of the nuclear hormone superfamily of receptors. There are three described isoforms: PPARα, PPARβ/δ and PPARγ (Issemann and Green, 1990). All three isoforms are expressed in the central nervous system (Moreno et al., 2004). Endogenous ligands at PPARs, include fatty acids (Marion-Letellier et al., 2016), serotonin derivatives (Waku et al., 2010a), and N-acylethanolamines (NAEs) including anandamide (AEA) (Bouaboula et al., 2005; Rockwell et al., 2006), N-palmitoylethanolamide (PEA) (LoVerme et al., 2005), and N-oleoylethanolamide (OEA) (Fu et al., 2003). PPARs are involved in many physiological processes and are targets for current in-use medicines for treatment of diabetes (Hong et al., 2018) and cholesterol lowering therapies (Fruchart et al., 2001). Moreover, studies suggest that the PPAR signalling system may act on pain (Okine et al., 2018), anxiety (Domi et al., 2016) and cognition (Varvel et al., 2007; Mazzola et al., 2009a; Babaei et al., 2012) processing.

PPARs are expressed in regions that play an important role in pain and fear/anxiety such as the prefrontal cortex (PFC) (Moreno et al., 2004; Okine et al., 2014; Warden et al., 2016), hippocampus (Moreno et al., 2004; Domi et al., 2016), amygdala (Warden et al., 2016), periaqueductal grey (PAG; Okine et al., 2017), spinal cord (Moreno et al., 2004) and dorsal root ganglion (Maeda et al., 2008; D'Agostino et al., 2009). Previous studies have shown that the selective activation of PPARα (LoVerme et al., 2006; Russo et al., 2007; Sagar et al., 2008; D'Agostino et al., 2009; Okine et al., 2014), PPARβ/δ (Gill et al., 2013a; Lyons et al., 2017), and PPARγ (Oliveira et al., 2007; Churi et al., 2008; Morgenweck et al., 2010; Hasegawa-Moriyama et al., 2012; Griggs et al., 2015; Mansouri et al., 2017b) has antinociceptive effects. Also, the pharmacological blockade of PPARγ expressed in the lateral PAG (lPAG) increases pain-related behaviour in Wistar-Kyoto (WKY), but not in Sprague-Dawley (SD) rats (Okine et al., 2017). The administration of PEA, a pan agonist at PPARs, suppresses nociception (LoVerme et al., 2006; D'Agostino et al., 2007, 2009b; Costa et al., 2008; Sasso et al., 2012; de Novellis et al., 2012; Bettoni et al., 2013; Wang et al., 2014b; Di Cesare Mannelli et al., 2015; Donvito et al., 2015, 2016; Okine et al., 2016).

Likewise, administration of the endogenous PPAR ligand OEA, and OEA-derived compounds, diminishes nociceptive behaviour (Vasconcelos et al., 2006; Suardíaz et al., 2007; Guida et al., 2015).

Fear is well recognised to modulate pain responding. An example of this is the phenomenon known as fear-conditioned analgesia (FCA), in which exposure to a fearful stimulus suppresses nociception. Different neuromodulators are involved in FCA such as the opioid, GABAergic, glutamatergic, monoaminergic, and endocannabinoid systems (Butler and Finn, 2009). Recent studies show that levels of AEA, PEA and OEA, three endogenous ligands at PPARs, are increased in the basolateral amygdala of rats expressing FCA (Olango et al., 2012; Rea et al., 2013), suggesting a possible role of PPARs in this potent form of endogenous analgesia. In turn, pain can regulate fear responses. Post-traumatic stress disorder (PTSD) symptoms tend to be more pronounced in patients with chronic pain (Asmundson et al., 2002). Additionally, patients with chronic pain are twice as likely to develop phobias (Pereira et al., 2017). There is some evidence that PPARγ blockade or knockout has anxiogenic effects in mice (Domi et al., 2016). However, whether PPARα or PPARβ/δ modulate anxiety or fear remains unexplored. Furthermore, the role of PPARs in reciprocal interactions between pain and fear has not yet been investigated.

In this chapter I investigate the hypothesis that the blockade of PPARs increases tonic inflammatory pain, and attenuate fear conditioned analgesia. Specifically, I examined the effects of the administration of GW6471 (PPARα antagonist), GSK0660 (PPARβ/δ antagonist), and GW9662 (PPARγ antagonist) on formalin-induced nociceptive behaviour, FCA, and conditioned-fear related behaviour in the presence of nociceptive tone in rats. I also determined whether any behavioural effects observed were accompanied by changes in levels of neurotransmitters, endocannabinoids, and NAEs within three key regions for fear and pain expression: basolateral amygdala (BLA), central nuclei of the amygdala (CeA), and ventral hippocampus (VH). Therefore, the specific aims of the two studies described in this chapter are:

- To determine if PPAR signalling plays a role in the expression of tonic persistent inflammatory pain and FCA by examining the effects of systemic administration of PPAR antagonists on formalin-evoked nociceptive behaviour and FCA in rats and associated alterations in levels of neurotransmitters, endocannabinoids, and NAEs in the BLA, CeA, and VH.

- To determine if PPAR signalling plays a role in the expression of conditioned fear in the presence of nociceptive tone by examining the effects of systemic administration of PPAR antagonists on fear-related behaviour, and associated alterations in levels of neurotransmitters, endocannabinoids, and NAEs in the BLA, CeA, and VH.
- To verify that the three antagonists used in the experiments can cross the blood-brain barrier and reach supraspinal regions.

2.2 Materials and Methods

2.2.1 Animals

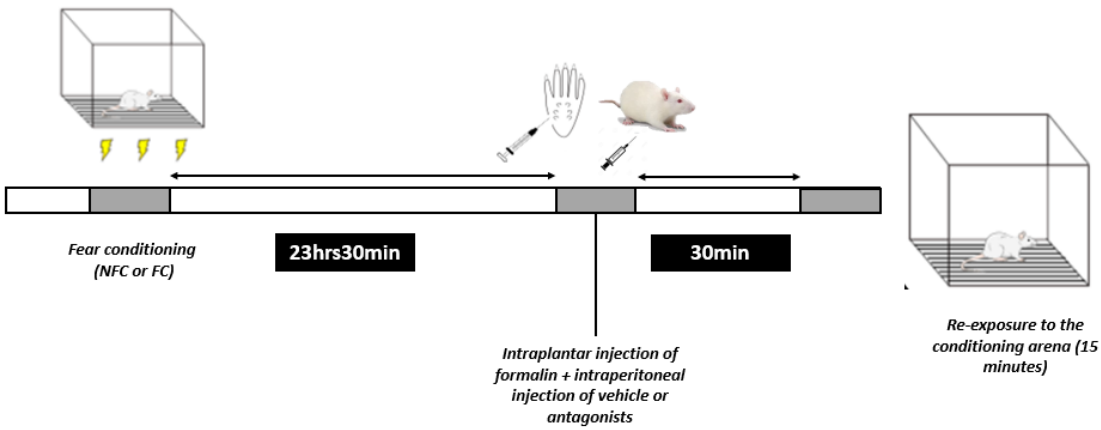
Experiments were carried out on a total of 54 (Experiment 1) and 36 (Experiment 2) adult male Sprague-Dawley rats (230-250g on arrival; Envigo UK, Bicester, England). The animals were maintained at controlled temperature (22± 2^{o}C) and humidity (45-55%) under standard lighting conditions (12:12h light-dark cycles, lights on from 07.00hrs). All experiments were carried out during the light phase. Animals were housed 2-3 per flat bottomed cage (L: 45 x H: 20 x W: 20cm) containing 3Rs paper bedding material (Fibrecycle Ltd., North Lincolnshire, United Kingdom) and sizzle nest material (LBS Biotechnology, Horley, United Kingdom) for the first week after arrival, and were posteriorly singly housed for the rest of the experiment. Food (14% Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, 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.

2.2.2 Experimental Procedures

The FCA paradigm was essentially as described in previous studies from our laboratory (Finn et al., 2004; Butler et al., 2008; Rea et al., 2018). There were two phases: conditioning (day 1) and test (day 2). On the conditioning day, rats were placed in a Perspex chamber (30 cm x 30 cm x 40 cm) and after 15 seconds they received the first of 10 footshocks (0.4mA, 1sec duration, LE85XCT Programmer and Scrambled Shock Generator; Linton Instrumentation, Norfolk, UK) spaced 60s apart. Thirty seconds after the last footshock, rats were returned to their home cage. The animals that belonged to the control group, that did not receive footshocks, were placed in the chamber for an equivalent time (10min). In Experiment 1, the animals were randomly assigned to one of 6 groups (n = 9 per group) – rats that received footshocks (FC) or no footshocks (NFC) treated with the PPARα antagonist GW6471, the PPARβ/δ antagonist GSK0660 or vehicle (1:1:8, ethanol: cremophor: 0.9% NaCl/saline). In Experiment 2, the animals were randomly assigned to one of 4 groups (n = 9 per group) – rats that received footshocks (FC) or no footshocks (NFC) treated with the PPARγ antagonist GW9662 or vehicle (1:1:8, ethanol: cremophor:

0.9% NaCl/saline). The sequence of testing was randomized to minimize any confounding effects of the order of testing.

The test day started 23hrs30min after the end of the conditioning phase. First, the rats received a 50µl injection of formalin (2.5% in saline) into the right hind paw under brief isoflurane anaesthesia (3% in O_2; 0.8L/min). Immediately after, still under anaesthesia, the animals in Experiment 1 received an intraperitoneal injection of either the PPARα antagonist GW6471, the PPARβ/δ antagonist GSK0660 or vehicle (volume of injection 3ml/kg), and the animals in Experiment 2 received an intraperitoneal injection of the PPARγ antagonist GW9662 or vehicle. After these injections, the rats were returned to their home cages. Thirty minutes later, or 24 hours after footshock, the rats were re-exposed to the conditioning chamber. A video camera located beneath the observation chamber was used to monitor animal behaviour. A 15min duration re-exposure was chosen on the basis of previous studies demonstrating that FCA peaks within this time period (Finn et al., 2004b; Roche et al., 2010; Ford et al., 2011; Rea et al., 2011b, 2013b). At the end of the test phase (45 min post-formalin injection), rats were killed by decapitation, brains were removed, snap frozen on dry ice and stored at −80°C. Formalin induced oedema was assessed by measuring the change in the diameter of the right hind paw measured immediately before, and 45 min after, formalin administration, using Vernier callipers.



2.2.3 Drugs

GW6471, GSK0660, and GW9662 (all obtained from Tocris Bioscience, Bristol, UK) were dissolved in a 1:1:8 (ethanol, cremophor; saline) vehicle solution. The doses of GW6471 (2mg/kg) and of GSK0660 (1mg/kg) were chosen based on studies in the literature demonstrating their efficacy in reversing the antinociceptive and neuroprotective effects of

PEA (Paterniti et al., 2013; Donvito et al., 2016). The dose of GW9662 (2mg/kg) was chosen based on the studies of Mansouri et al (2017), Griggs et al (2015) and Morgenweck et al (2013) showing that this dose was effective in reversing antinociceptive effects of pioglitazone. Formalin was prepared from a 37% stock solution (Sigma-Aldrich, Dublin, Ireland) diluted in sterile saline.

2.2.4 Behavioural analysis

Behaviour was analysed using Ethovision 11.5 XT software package (Noldus Technology, Wageningen, Netherlands). A trained observer blind to the experimental conditions assessed behaviour, including: (1) freezing duration (defined as the absence of visible movement except that needed for respiration), (2) duration of walking, (3) duration of grooming, and (4) duration of rearing. Moreover, formalin-evoked nociceptive behaviour was scored according to the composite pain scoring (CPS) technique described by Watson et al (1997) in which pain behaviours are classified as time spent raising the formalin-injected paw (P1), and holding, licking, biting, shaking or flinching the injected paw (P2). Thus, we obtain a CPS value from the equation [CPS = (P1+2(P2))/ (total duration of trial)].

2.2.5 Brain extraction

After decapitation, the optic ridge between the eyes was broken with the use of rongeurs. Then, a cut in the skin was made, from the eyes until the neck, and the skull exposed. Any remaining skin, muscles and fascia was scraped away. With the help of a small scissors, the bone was removed from the foramen magnum and one of the tips of a small scissors made pressure from the inside, breaking the posterior part of the cranium. From the upper edge of the foramen magnum, the pressure and cut were kept upwardly (from the posterior to the anterior part of the skull) and outside ward (from the inside to the outside of the skull), always carefully in order to preserve the brain tissue underneath. The occipital, parietal, temporal and frontal divisions of the cranium could be removed once the process was over. If any remaining part of the skull bone (normally the frontal lobe) was left, the same pressure approach with the small scissors was taken. Once exposed, the brain was collected with the help of a spatula. The spatula was placed in the lateral part of one of the brain hemispheres and, carefully, slid down between the bone and the brain. This process was repeated for the other hemisphere. Then, the brain could be carefully lifted, minding the attachments made by the olfactory nerves in the upper dorsal, and optical and trigeminal nerves in the low dorsal part of the brain. Once free and movable, the brains were snap-frozen on dry ice and stored at -80^{o}C.

2.2.6 Cryo-sectioning and tissue microdissection

Frozen coronal brain sections of 150 µm thickness containing the basolateral amygdala (BLA), central nuclei of the amygdala (CeA), and ventral hippocampus (VH) were cut on a cryostat (Leica Biosystems, Wetzlar, Germany), and were punch-dissected as previously described (Olango et al., 2012; Rea et al., 2014), using cylindrical brain punchers (Harvard Apparatus, MA, USA) with an internal diameter of 0.50mm for the different amygdalar nuclei, at the following rostro-caudal levels (obtained from the rat brain atlas by Paxinos and Watson, 2006: (BLA) Bregma, - 2.12 – -3.30mm, (CeA) Bregma, - 2.12 – -3.30mm. A cylindrical brain puncher with an internal diameter of 0.75mm was used to collect the VH at the following rostro-causal level: (VH) Bregma, -7.3 - -8.3mm). Additionally, in order to evaluate possible lateralisation effects, the punches were separately collected for right and left hemispheres. The punch-dissected tissue was weighed (BLA - 1.72 ± 0.1mg; CeA - 2.1±0.3mg; VH – 4.875 ± 1.8mg) and stored at -80^{o}C prior to measurement of AEA, PEA, OEA, 2-AG, and neurotransmitter levels by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2.2.7 Measurement of endocannabinoids, NAEs and neurotransmitters in discrete brain regions using liquid chromatography – tandem mass spectrometry (LC-MS/MS)

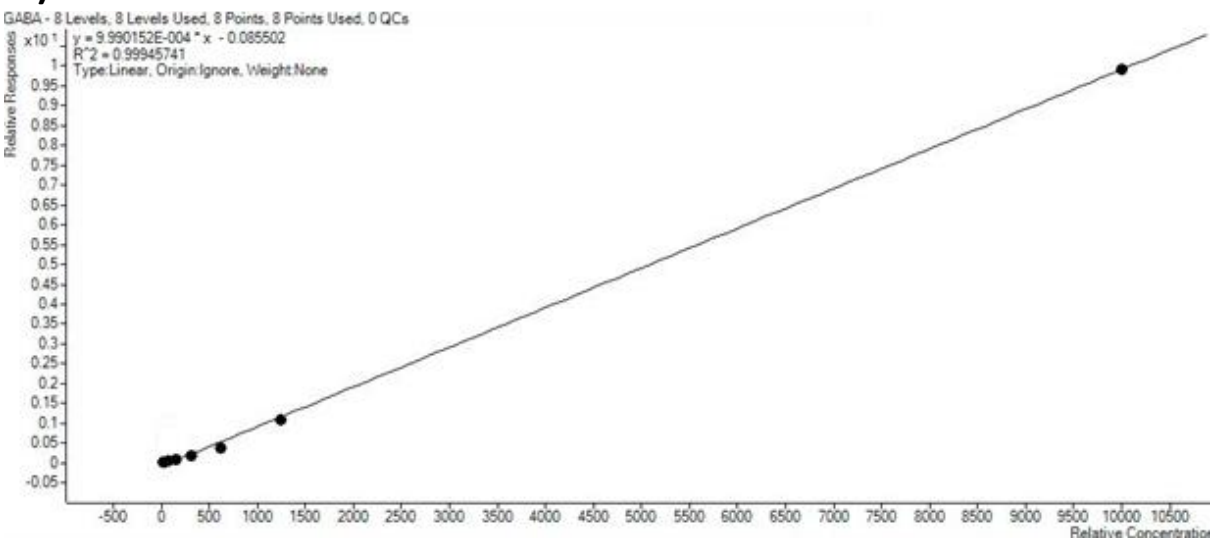
Tissue extraction was carried out using the following method: each punch-dissected brain sample was homogenised for 4-6s ultrasonic homogeniser/sonicator (Mason, Dublin, Ireland) in a mixture containing 200µl of deuterated internal standards for endocannabinoids (0.48nmol/50ng of 2-AG-d8 and 0.014nmol/2.5ng of AEA-d8) and NAEs (0.015nmol/2.5ng of OEA-d2 and 0.016nmol/2.5ng of PEA-d4), and 10µl of deuterated internal standards for neurotransmitters (5µg/0.048µmol of GABA-d6, 5µg/0.033µmol of Glutamate d-5, 1ng/0.006nmol of dopamine-d-4, and 1ng/0.005nmol of serotonin-d-4) and immediately kept on ice. The final volume was made up to 260µl prior to sonication by adding 50µl of 100% acetonitrile. Deuterated and non-deuterated endocannabinoids were purchased from Cayman Chemicals (Biosciences, UK). Non-deuterated neurotransmitters were purchased from from Sigma Chemicals (Ireland): 2129-GABA, G1251-glutamate, H8502-dopamine, and H9523-serotonin. Deuterated neurotransmitters for GABA, glutamate and dopamine were acquired from CDN isotopes (Canada) (D1828-GABA (D6), D2193-glutamate (D5), D1540-dopamine (D4)). The deuterated serotonin was procured from Alsachim (France) M760-serotonin (D4).

Samples were kept on ice during the procedure. The homogenates were centrifuged at 11000g for 15min at 4°C (Hettich centrifuge Mikro 22R, Germany). Immediately after, the supernatant was collected and 40µl was transferred to a HPLC vial. The standard curve was constructed using serial 1/2 dilution by adding 50µl of a mixture of non-deuterated endocannabinoids and NAEs (25ng for PEA, OEA and AEA + 250ng for 2-AG) and 10µl of a mixture of non-deuterated neurotransmitters (100µg of glutamate and GABA, 10ng each of dopamine, noradrenaline and serotonin) to 40 µl of acetonitrile in tube #10, vortex-mixing, then collecting 50µl and transferring to the next tube (#9) containing 50µl acetonitrile. The process was repeated until tube #1, when 50µl of the final volume was discarded, in order to keep the volumes between tubes consistent. Thus, all 10 tubes had 50µl of a mixture of endocannabinoids and neurotransmitters. All standard curve tubes were spiked with 200µl of deuterated endocannabinoid/NAE mixture (2.5ng deuterated PEA, OEA and AEA and 50ng deuterated 2-AG as internal standards) and 10µl of deuterated neurotransmitter mixture (5µg of glutamate and GABA, and 1ng each of dopamine, and serotonin). A double blank (100% acetonitrile) was also included in between each standard point during the run to minimise the risk of analyte carryover from standard to standard at the upper range of the curve and five double blanks were included after the highest concentration point on the curve to avoid carryover onto the samples. A quality control (QC) sample was prepared from the whole rat brain homogenate, using the same protocol described for the punches, and was included with each run to allow for monitoring of inter-runs variability. The QC was added after all the samples, in the end of the run.

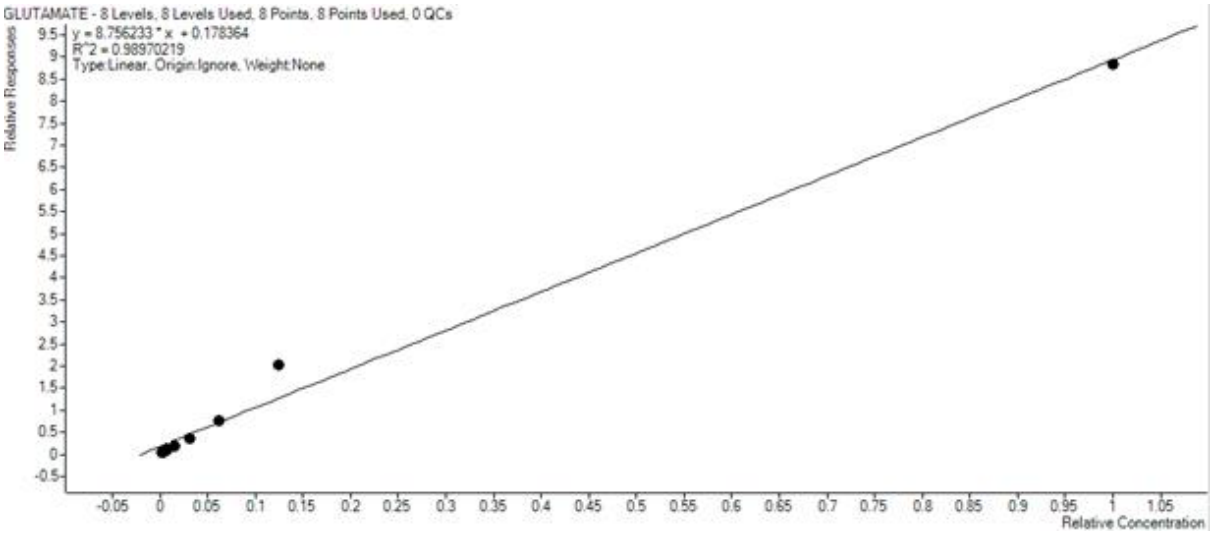
Mobile phases consisted of (1) high pressure liquid chromatography (HPLC) grade water with 0.1% (v/v) formic acid and (2) acetonitrile with 0.1% (v/v) formic acid for the initial three minutes with a flow rate of 0.2ml/min using a Waters Atlantis T3 column (3µm particles, 100mm length, 2.1mm diameter; Waters, UK). Reversed-phase gradient elution was initiated at 2% acetonitrile for the first three minutes, set to 65% acetonitrile at 3.1 minutes for one minute and then ramped linearly up to 100% acetonitrile at 8 minutes and held at 100% acetonitrile until 16 minutes. At 16.1min, the gradient returned to initial conditions for a further 12 min to re-equilibrate the column. The total run time was 28min. Under these conditions, GABA, glutamate, dopamine and serotonin, AEA, 2-AG, PEA, OEA, eluted at the following retention times: 1.3min, 1.4min, 1.4min, 1.8min, 13.5min, 13.9min, 14.2min and 14.6min respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple

quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Instrument conditions were optimised for each analyte by infusing standards separately. Quantitation of target endocannabinoids and neurotransmitters was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M + H+] of the analytes of interest and the deuterated form of the internal standard (MRM spectra and mass-to-charge (m/z) ratios of each analyte of interest and its corresponding internal standard are displayed in Fig 2.1. Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was calculated using Masshunter Quantitative Analysis Software (Agilent Technologies Ltd, Cork, Ireland). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. The values obtained from the Masshunter Quantitative Analysis Software are initially expressed in ng per mg of tissue by dividing by the weight of the punched tissue. To express values as nmol or pmols per mg the corresponding values are then divided by the molar mass of each analyte expressed as ng/nmole or pg/pmole.

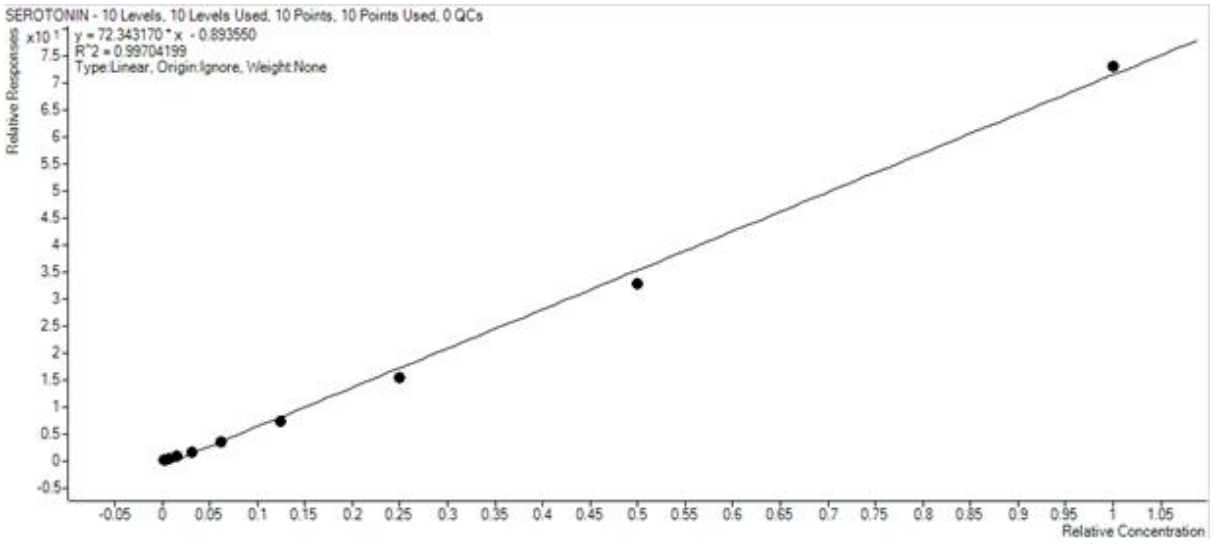
A)



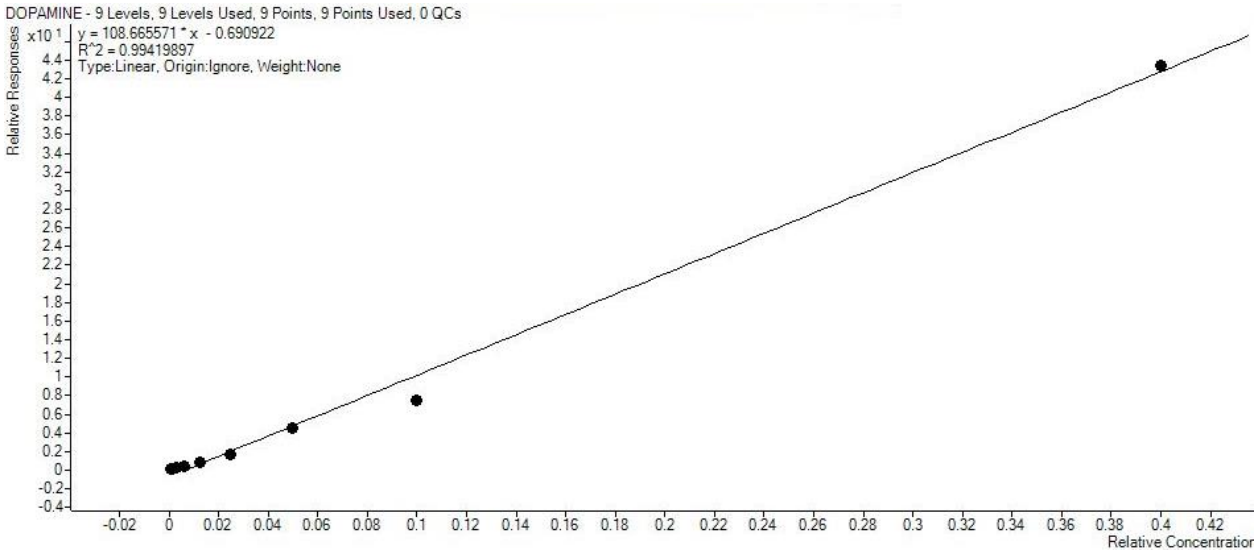
B)


GLUTAMATE - 8 Levels, 8 Levels Used, 8 Points, 8 Points Used, 0 QCs
y = 8.756233 * x + 0.178364
R^2 = 0.98970219
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Relative Concentration

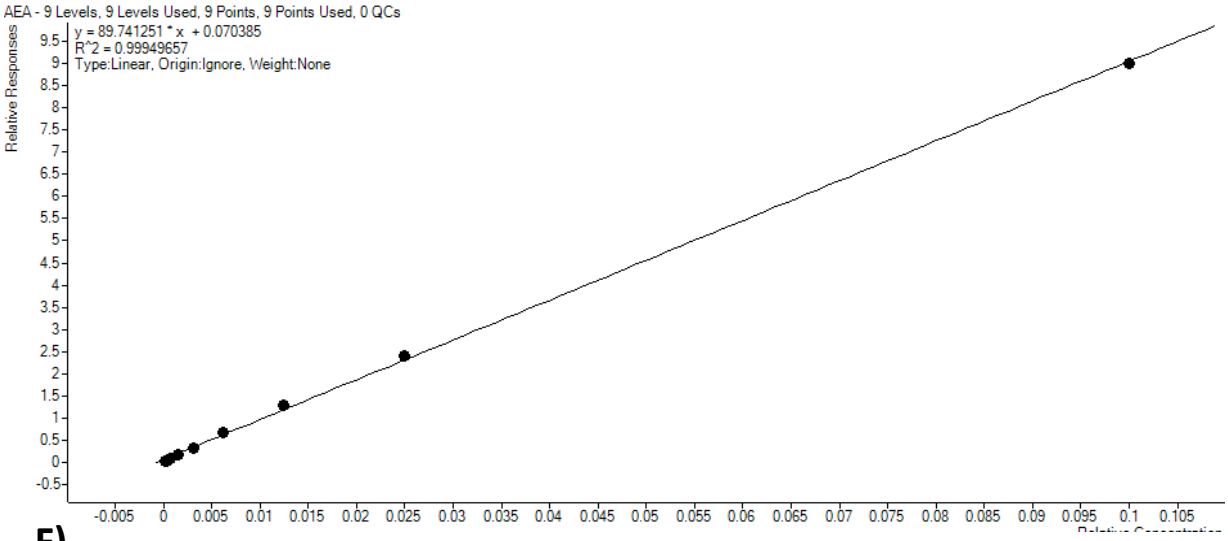
C)


SEROTONIN - 10 Levels, 10 Levels Used, 10 Points, 10 Points Used, 0 QCs
x10 1
y = 72.343170 * x - 0.893550
R^2 = 0.99704199
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Relative Concentration

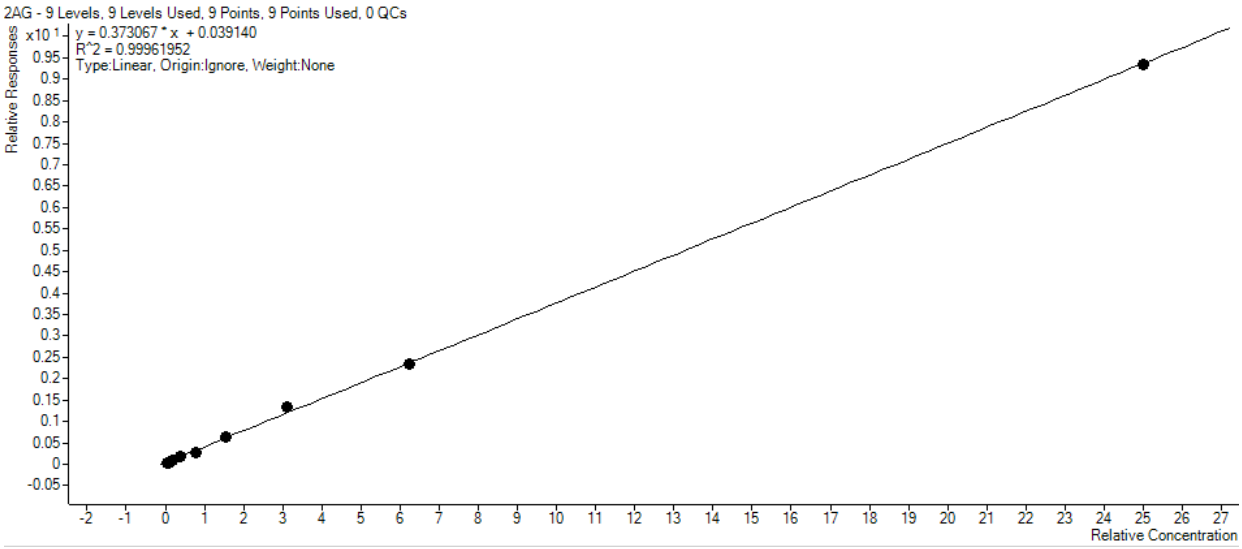
D)


DOPAMINE - 9 Levels, 9 Levels Used, 9 Points, 9 Points Used, 0 QCs
x10 1
y = 108.665571 * x - 0.690922
R^2 = 0.99419897
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
Relative Concentratior

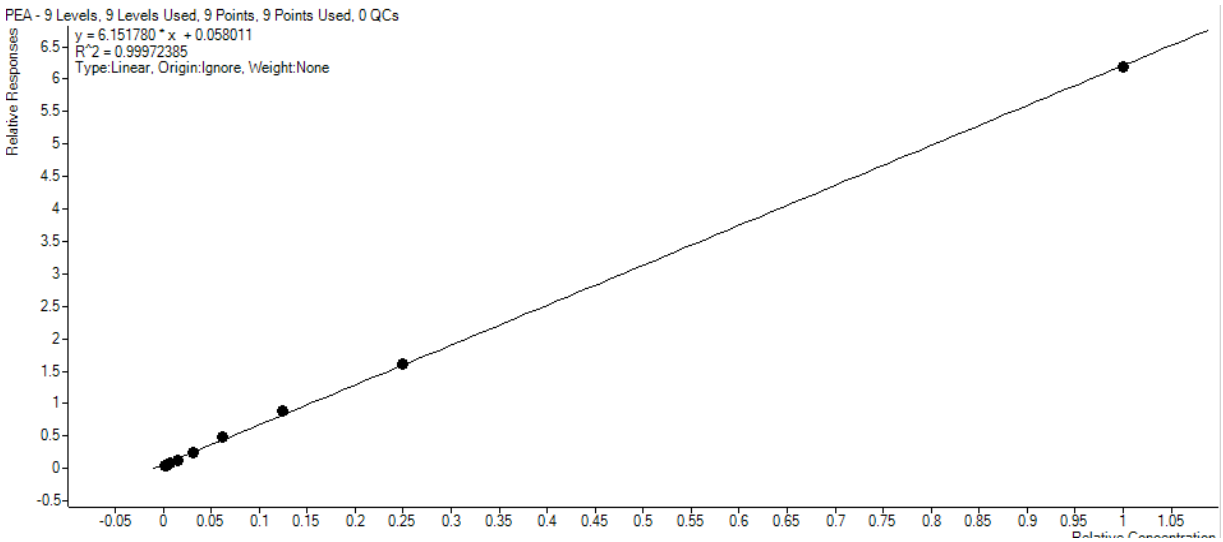
E)


AEA - 9 Levels, 9 Levels Used, 9 Points, 9 Points Used, 0 QCs
y = 89.741251 * x + 0.070385
R^2 = 0.99949657
Type:Linear, Origin:Ignore, Weight:None
Relative Responses

F)


2AG - 9 Levels, 9 Levels Used, 9 Points, 9 Points Used, 0 QCs
y = 0.373067 * x + 0.039140
R^2 = 0.99961952
Type:Linear, Origin:Ignore, Weight:None
Relative Responses
x10 1
Relative Concentration

G)



H)

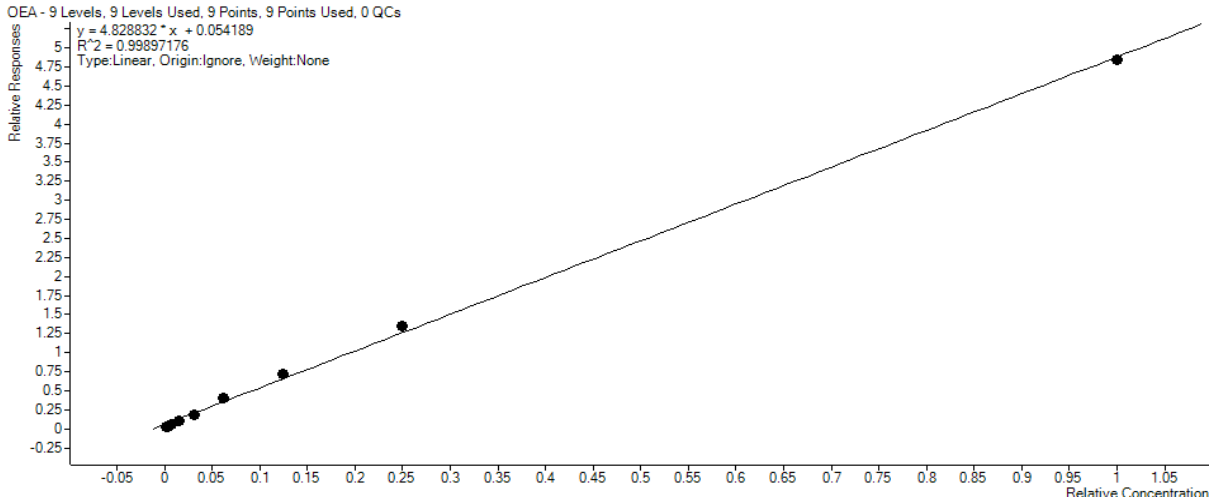
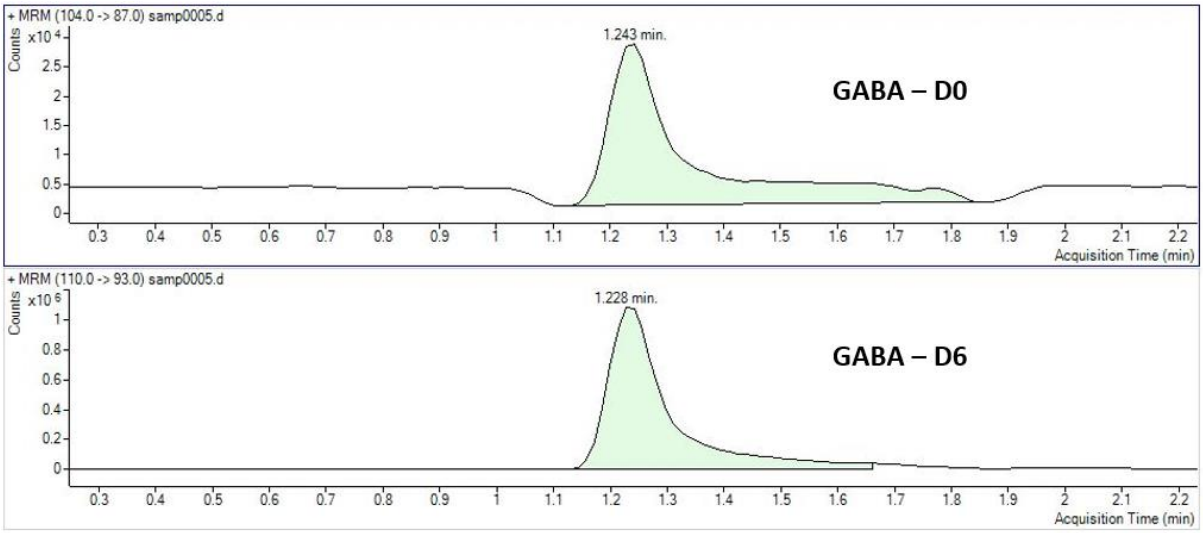
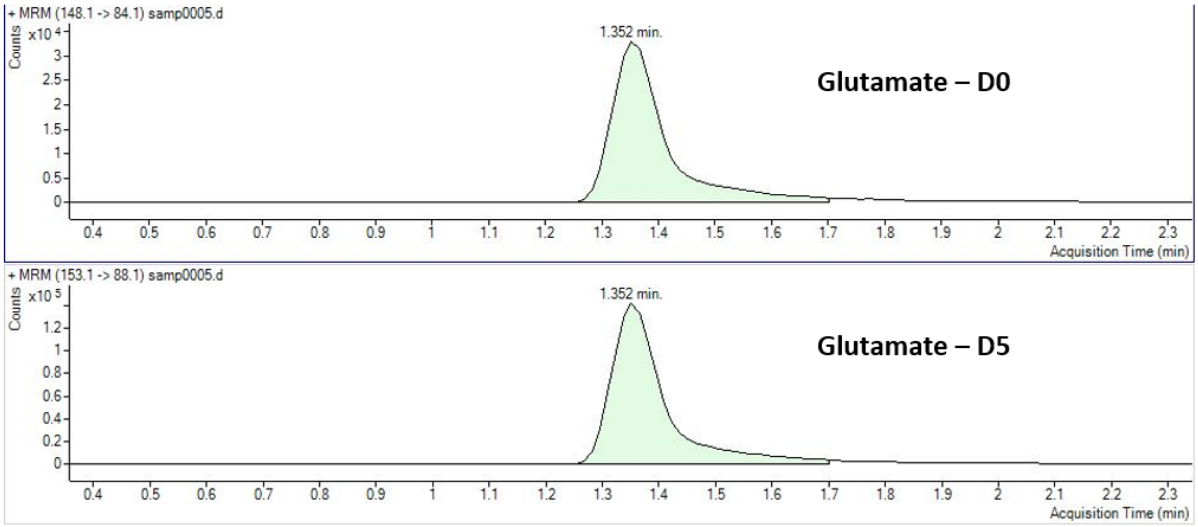


Figure 2.1: Example 9- or 10-point standard curves for GABA (A), glutamate (B), serotonin (C), dopamine (D), AEA (E), 2-AG (F), PEA (G), and OEA (H). Plot of relative response (y-axis) versus Relative Concentration (x-axis). Relative response is the ratio of peak area of undeuterated analyte to peak area of deuterated analyte. Relative concentration is the ratio of amount in ng of undeuterated analyte to the amount in ng of deuterated analyte.

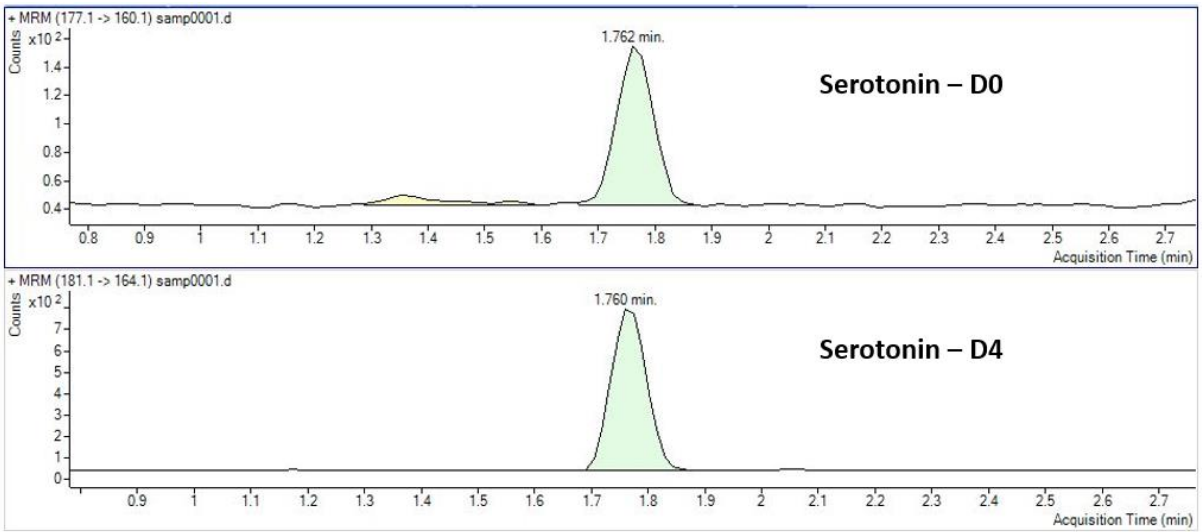
A)


+ MRM (104.0 -> 87.0) samp0005.d
Counts
x10 4
1.243 min.
GABA – D0
Acquisition Time (min)
+ MRM (110.0 -> 93.0) samp0005.d
Counts
x10 6
1.228 min.
GABA – D6
Acquisition Time (min)

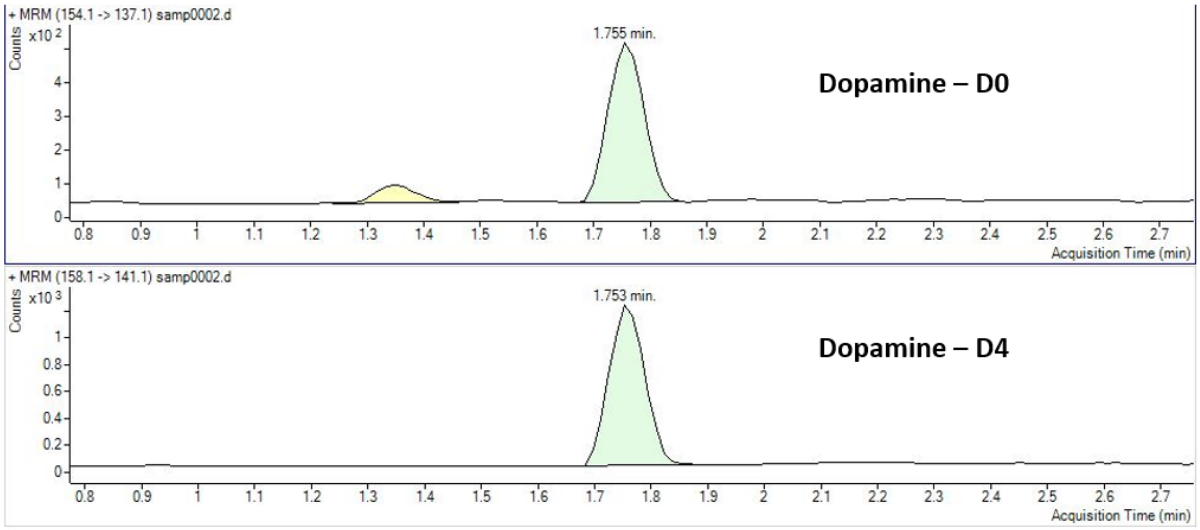
B)


+ MRM (148.1 -> 84.1) samp0005.d
Counts
x10 4
1.352 min.
Glutamate – D0
Acquisition Time (min)
+ MRM (153.1 -> 88.1) samp0005.d
Counts
x10 5
1.352 min.
Glutamate – D5
Acquisition Time (min)

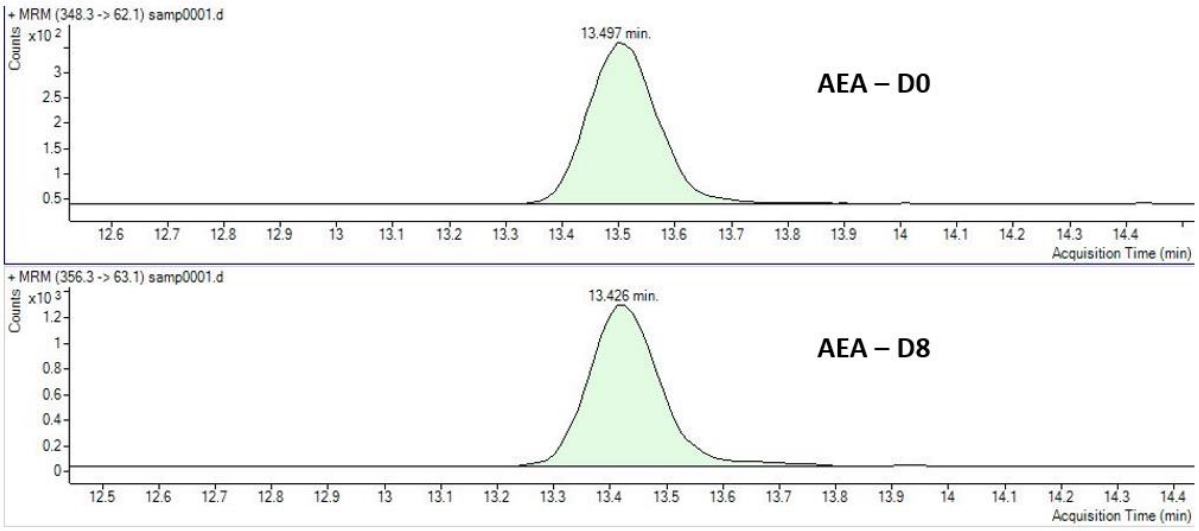
C)


+ MRM (177.1 -> 160.1) samp0001.d
Counts
x10 2
1.762 min.
Serotonin – D0
Acquisition Time (min)
+ MRM (181.1 -> 164.1) samp0001.d
Counts
x10 2
1.760 min.
Serotonin – D4
Acquisition Time (min)

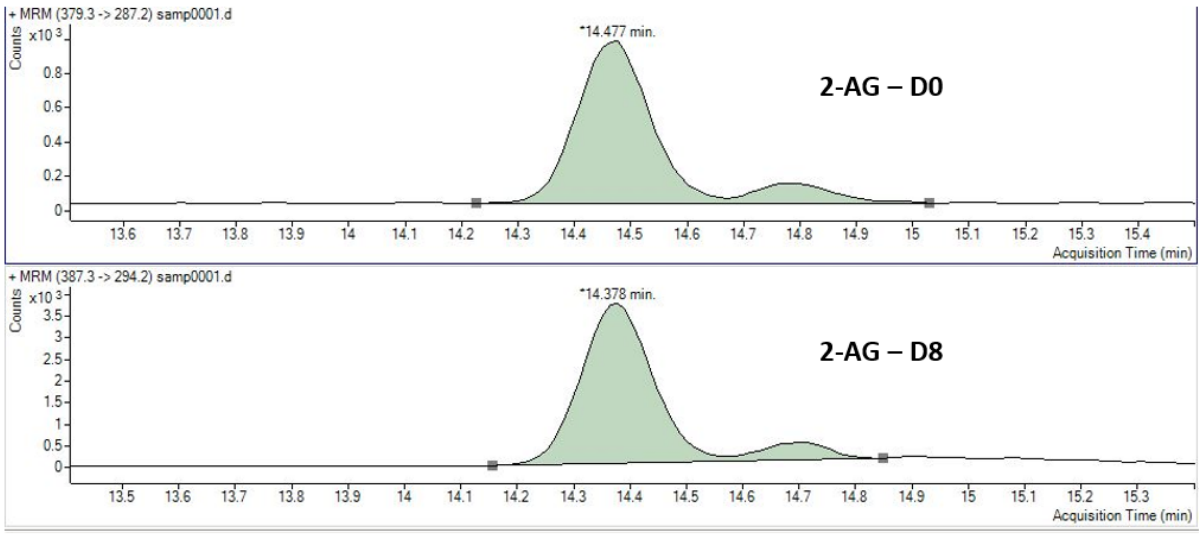
D)


+ MRM (154.1 -> 137.1) samp0002.d
Counts
x10 2
1.755 min.
Dopamine – D0
Acquisition Time (min)
+ MRM (158.1 -> 141.1) samp0002.d
Counts
x10 3
1.753 min.
Dopamine – D4
Acquisition Time (min)

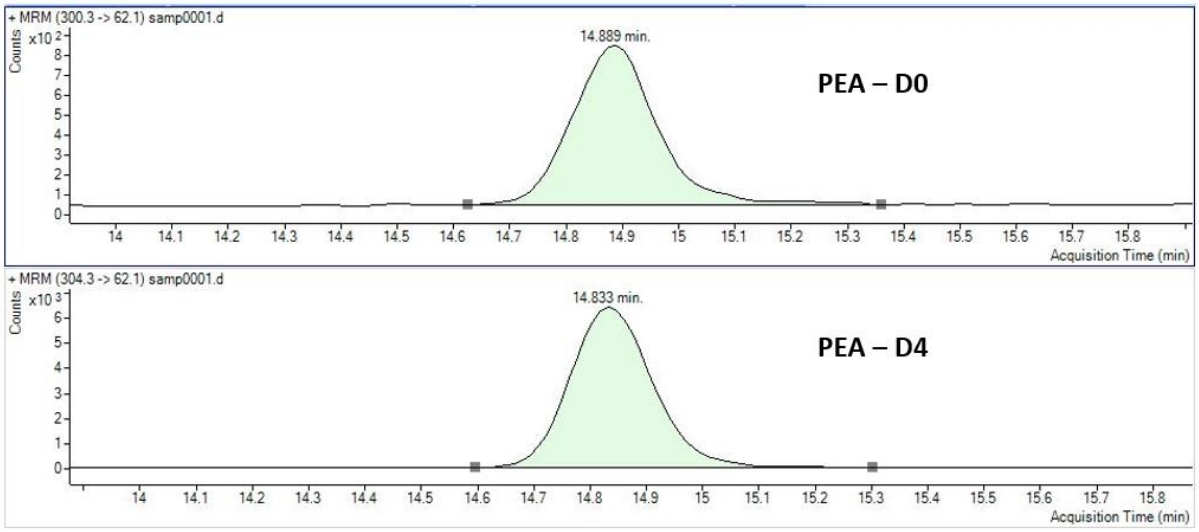
E)


+ MRM (348.3 -> 62.1) samp0001.d
Counts
x10 2
13.497 min.
AEA – D0
Acquisition Time (min)
+ MRM (356.3 -> 63.1) samp0001.d
Counts
x10 3
13.426 min.
AEA – D8
Acquisition Time (min)

F)


+ MRM (379.3 -> 287.2) samp0001.d
Counts
x10 3
*14.477 min.
2-AG – D0
Acquisition Time (min)
+ MRM (387.3 -> 294.2) samp0001.d
Counts
x10 3
*14.378 min.
2-AG – D8
Acquisition Time (min)

G)



H)

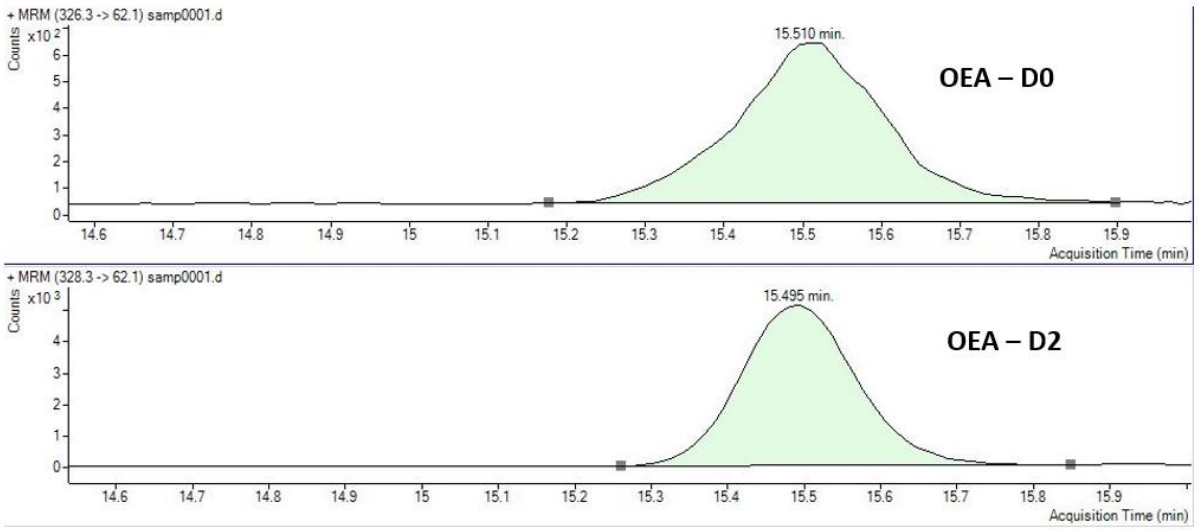


Figure 2.2: Chromatograms of neurotransmitters (GABA – A; Glutamate – B; Serotonin – C; Dopamine – D), endocannabinoids (AEA, 2-AG – E, F), and related NAEs (PEA – G; OEA – H). Top image displays the undeuterated target analytes and bottom image is the deuterated internal standard.

2.2.8 Detection of drugs in brain tissue

2.2.8.1 Detection of GW6471 and GSK0660

Punched dorsal hippocampus (DH) tissue samples (4.85±1.2mg) were sonicated in a mixture containing 200µl of deuterated internal standards for endocannabinoids (0.48nmol of 2-AG-d8 and 0.014nmol of AEA-d8) and NAEs (0.015nmol of OEA-d2 and 0.016nmol of PEA-d4), 10µl of deuterated internal standards for neurotransmitters (5µg/0.048µmol of GABA-d6, 5µg/0.033µmol of Glutamate d-5, 1ng/0.006nmol of dopamine-d-4, and 1ng/0.005nmol of serotonin-d-4) and 50µl of 100% acetonitrile using an ultrasonic homogeniser/sonicator (Mason, Dublin, Ireland). Samples were kept on ice during the procedure. The homogenates were centrifuged at 14000g for 15min at 4°C. Immediately after, the supernatant was collected and 40µl of it was transferred to a HPLC vial. Mobile phases consisted of (1) high pressure liquid chromatography (HPLC) grade water with 0.1% formic acid and (2) acetonitrile, with a flow rate of 200µl/min using an Agilent Zorbax reverse phase C18 RRHT column, 50mm length, 2.1mm diameter, 1.8um particles at 40°C. Analytes were detected using jet stream-positive ionisation with an Agilent triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland) interfaced with an Agilent 1100 HPLC system. Reversed-phase gradient elution began initially at 75% acetonitrile for the first minute and then ramped by 5min to 100% acetonitrile. The nebulizer gas pressure was set at 45PSI (Vcap of 3500) and the source temperature at 300°C. Sheath Gas flow and temperature was 11L/min and 250°C respectively. GW6471 (Fig 2.3A) was monitored by SRM from 620.2 to 391.2 m/z (at a collision energy setting of 35V) and GSK0660 (Fig 2.3B) was monitored from 419.0 to 214.0 m/z (at collision energy of 5V). The fragmentor voltage was set at 200V.

2.2.8.2 Detection of GW9662

Punched DH samples (4.785±1.15mg) were sonicated in a mixture containing 200µl of deuterated internal standards for endocannabinoids (0.48nmol of 2-AG-d8 and 0.014nmol of AEA-d8) and NAEs (0.015nmol of OEA-d2 and 0.016nmol of PEA-d4), 10µl of deuterated internal standards for neurotransmitters (5µg/0.048µmol of GABA-d6, 5µg/0.033µmol of Glutamate d-5, 1ng/0.006nmol of dopamine-d-4, and 1ng/0.005nmol of serotonin-d-4) and 50µl of 100% acetonitrile using an ultrasonic homogeniser/sonicator (Mason, Dublin, Ireland). Samples were kept on ice during the procedure. The homogenates

were centrifuged at 11000g for 15min at 4°C. Immediately after, the supernatant was collected and 40µl of it was transferred to a HPLC vial. Mobile phases consisted of (1) high pressure liquid chromatography (HPLC) grade water with 0.1% formic acid and (2) acetonitrile, with a flow rate of 200µl/min using an Agilent Zorbax reverse phase C18 RRHT column, 50mm length, 2.1mm diameter, 1.8um particles at 40°C. Analytes were detected using electrospray-positive ionisation with an Agilent triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland) interfaced with an Agilent 1100 HPLC system. Reversed-phase gradient elution began initially at 2% acetonitrile for the first minute and then ramped at 6min to 100% acetonitrile. The nebulizer gas pressure was set at 45PSI (Vcap of 3500) and the source temperature at 300°C. Sheath Gas flow and temperature was 11L/min and 250°C respectively. GW9662 (Fig 2.4) was monitored by SRM from 275.2 to 156.1 m/z (at a collision energy setting of 35V). The fragmentor was set at 200V.

Figure 2.3: Detection of GW6471 (A) and GSK0660 (B) in DH punches. Analytes were detected using jet stream-positive ionisation with an Agilent triple quadrupole 6460 mass spectrometer interfaced with an Agilent 1100 HPLC system. The top chromatogram represents the detection of GW6471 at 620.2 to 391.2 m/z; the middle chromatogram represents the detection of GSK0660 at 419.0 to 214.0 m/z; the two bottom chromatograms represent the absence of the those transitions at the same retention times, indicating absence of the drugs (e.g. vehicle-treated subjects).

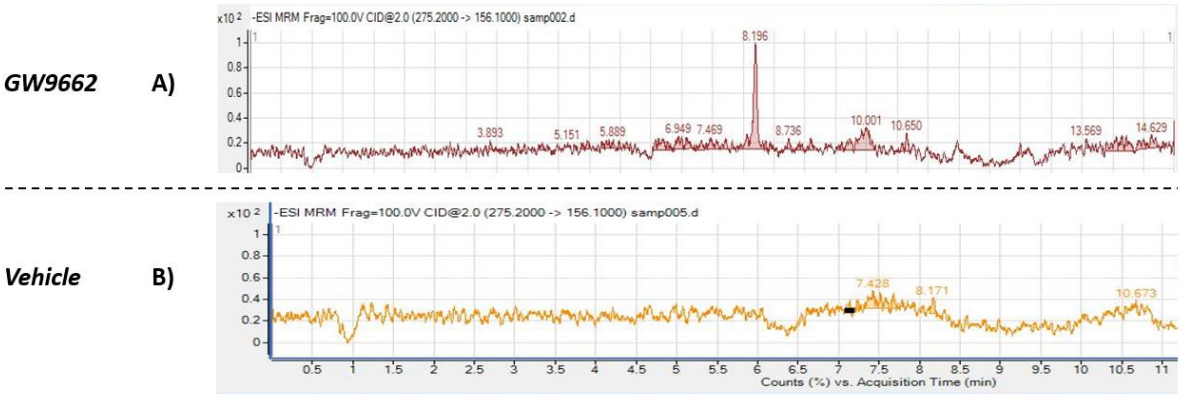


Figure 2.4: Detection of GW9662 (A) in DH punches. Analytes were detected using jet stream-positive ionisation with an Agilent triple quadrupole 6460 mass spectrometer interfaced with an Agilent 1100 HPLC system. The top chromatogram represents the detection of GW9662 at 275.2 to 156.10 m/z and the bottom chromatograms represent the absence of the that transition at the same retention time, indicating absence of the drug (e.g. vehicle-treated subjects).

2.2.9 Statistical Analysis

The SPSS 21.0 statistical package was used to analyse data. Normality was assessed using Shapiro-Wilk test and homogeneity of variance was checked using Levene's test. Behavioural data were analysed using two-factor analysis of variance (Two-way ANOVA), with factors being fear-conditioning and treatment, or repeated measures ANOVA when appropriate (e.g. when the data were analysed and presented in time bins). Neurochemical data were analysed using three-factor analysis of variance (Three-way ANOVA), with factors being fear conditioning, treatment, and side (Right [ipsilateral] or left [contralateral], with respect to the formalin injection). *Post hoc* pairwise comparisons were made with Student Newman-Keuls test when appropriate. If data were found to be non-parametric, three transformations were applied, in this order: square root of the data values, log of the data values, and ranking of the data values. Also, it was checked if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular data set being analysed (Thunder et al., 2007). If data were still deemed non-parametric after these transformations and tests, they were analysed using Kruskal-Wallis analysis of variance and *post hoc* analysis performed using Dunn's test when appropriate. When repeated measures were non-parametric distributed, data were analysed using Friedman's and Kruskal Wallis tests followed by Dunn's *post hoc* test if applicable. Data were

considered significant when $p<0.05$. Data are expressed as group means ± standard error of the mean (S.E.M.) when parametric and as median with interquartile range when non-parametric.

Possible presence of outliers was checked by assessing the distribution of data. In case the data 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.

Results

2.3.1 Experiment 1

2.3.1.1 Effects of systemic administration of GW6471 and GSK0660 on formalin-evoked nociceptive behaviour and FCA

Intra-plantar administration of formalin into the right hind paw produced robust nociceptive behaviour as evidenced by the composite pain score. Kruskal-Wallis test revealed a significant difference among groups (χ^2 (5) = 40.62, p<0.001) (Figure 2.5A). *Post hoc* analysis with Dunn's test revealed that formalin-evoked nociceptive behaviour was significantly lower in all fear-conditioned groups compared with their non-fear-conditioned counterparts, confirming expression of FCA (FC Vehicle vs NFC Vehicle [**p<0.001], FC GW6471 vs NFC GW6471 [$^{\#\#}$p<0.001], and FC GSK0660 vs NFC GSK0660 [$^{\$\$}$p<0.001]). Neither GW6471 nor GSK0660 had any significant effect on formalin-evoked nociceptive behaviour in non-fear-conditioned or fear-conditioned rats (i.e. no effect on FCA).

The analysis of pain 1 (see definition in the section 2.2.4) duration (Figure 2.5B) with Kruskal Wallis test revealed a significant difference among groups (χ^2 (5) = 40.22, p<0.001). *Post hoc* analysis with Dunn's test revealed that formalin-evoked nociceptive behaviour was significantly lower in all fear-conditioned groups compared with their non-fear-conditioned counterparts, confirming expression of FCA (FC Vehicle vs NFC Vehicle groups [*p<0.001], FC GW6471 vs NFC GW6471 [$^{\#\#}$p<0.001], and FC GSK0660 vs NFC GSK0660 [$^{\$\$}$p<0.001]). There were no significant effects of either GW6471 or GSK0660 on formalin-evoked nociceptive behaviour in NFC or FC rats.

The analysis of pain 2 (see definition in the section 2.2.4) duration (Figure 2.5C) with Kruskal Wallis test revealed a significant difference among groups (χ^2 (5) = 42.98, p<0.001). *Post hoc* analysis with Dunn's test revealed that formalin-evoked nociceptive behaviour was significantly lower in all fear-conditioned groups compared with their non-fear-conditioned counterparts, confirming expression of FCA (FC Vehicle vs NFC Vehicle groups [**p<0.01], FC GW6471 vs NFC GW6471 [$^{\#\#}$p<0.001], and FC GSK0660 vs NFC GSK0660 [$^{\$\$}$p<0.001]).

Friedman's test showed a significant effect of time [(χ^2 (4) = 11.025, p<0.001] on CPS values. Post hoc analysis with Wilcoxon test revealed a difference between times 1-3 and 10-12 (p<0.01), 1-3 and 13-15 (p<0.01), 4-6 and 10-12 (p<0.01), and 7-9 and 10-12

($p<0.05$). Kruskal Wallis comparisons revealed a significant difference between the groups in each time bin [($\chi^2_{1\text{-}3}$ (5) = 39.281, ***$p<0.001$; $\chi^2_{4\text{-}6}$ (5) = 41.565, ***$p<0.001$; $\chi^2_{7\text{-}9}$ (5) = 40.959, ***$p<0.001$; $\chi^2_{10\text{-}12}$ (5) = 41.102, ***$p<0.001$; $\chi^2_{13\text{-}15}$ (5) = 36.379, *** $p<0.001$)] in CPS. (Figure 2.6). *Post hoc* analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in the FC Vehicle group compared to NFC Vehicle [(Time $_{1\text{-}3}$, $p<0.01$; Time $_{4\text{-}6}$, $p<0.01$; Time $_{7\text{-}9}$, $p<0.001$; Time $_{10\text{-}12}$, $p<0.01$; Time $_{13\text{-}15}$, $p<0.05$], in the FC GW6471 group compared to NFC GW6471 [(Time $_{1\text{-}3}$, $p<0.01$; Time $_{4\text{-}6}$, $p<0.01$; Time $_{7\text{-}9}$, $p<0.001$; Time $_{10\text{-}12}$, $p<0.001$; Time $_{13\text{-}15}$, $p<0.01$], and in the FC GSK0660 group compared to NFC GSK0660 [(Time $_{1\text{-}3}$, $p<0.05$; Time $_{4\text{-}6}$, $p<0.01$; Time $_{7\text{-}9}$, $p<0.01$; Time $_{10\text{-}12}$, $p<0.05$; Time $_{13\text{-}15}$, $p<0.01$]. Neither GW6471 nor GSK0660 had any significant effect on formalin-evoked nociceptive behaviour in non-fear-conditioned or fear-conditioned rats (i.e. no effect on FCA).

Two-way ANOVA revealed no significant effect of fear-conditioning [$F(1, 48) = 0.6788$, $p>0.05$] or treatment [$F(2, 48) = 1.229$, $p>0.05$] on formalin-induced paw oedema (Figure 2.7).

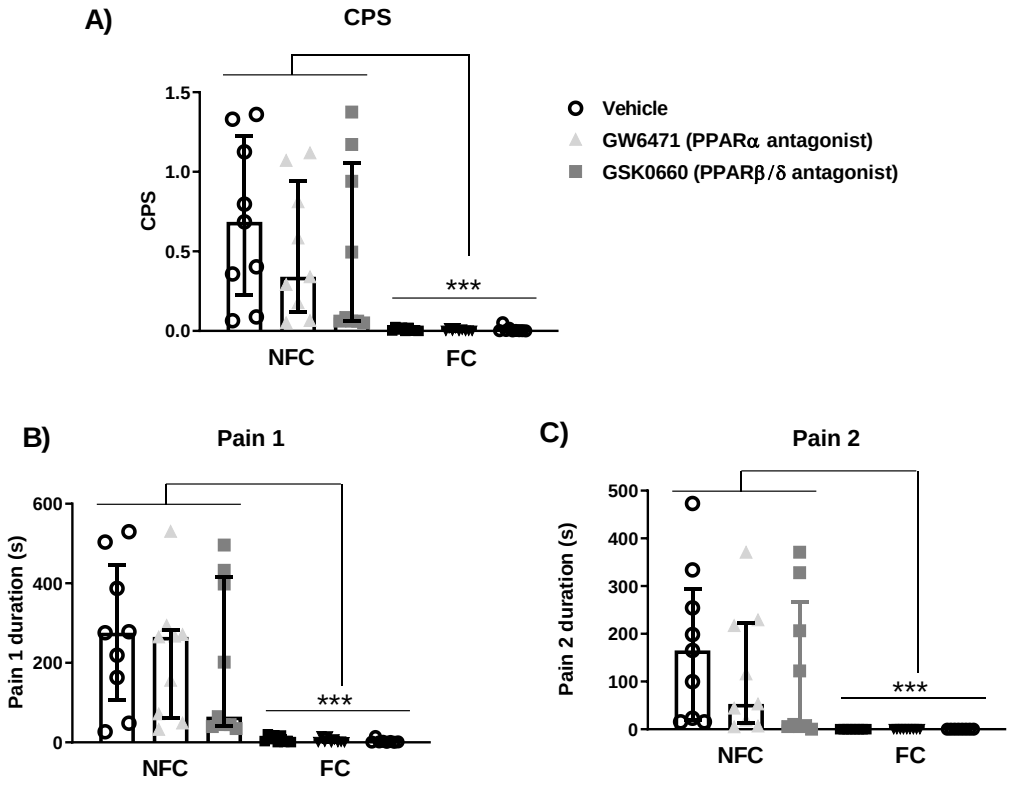


Figure 2.5: Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on formalin-evoked nociceptive behaviour in non-fear conditioned (NFC) and fear conditioned (FC) rats. *Post hoc* analysis with Dunn's test revealed a significant difference between formalin-injected FC groups and their NFC counterparts (***$p<0.05$) in CPS (Figure A), Pain 1 (Figure B) and Pain 2 (Figure C) values. Data are expressed as median with interquartile range (n=9 per group).

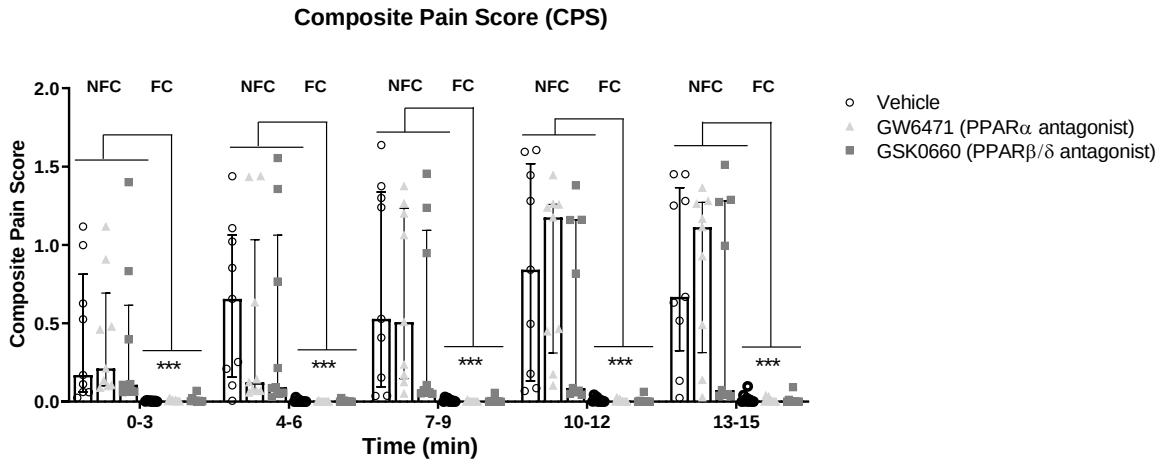


Figure 2.6: Temporal profile of the effects of systemic administration of selective PPARα and PPARβ/δ antagonists on formalin-evoked nociceptive behaviour in non-fear conditioned (NFC) and fear conditioned (FC) rats. Post hoc analysis with Dunn's test revealed a significant difference between NFC groups and their FC counterparts (***$p<0.05$ in all time bins. Data are expressed in 3-min bins (median with interquartile range; n=9 rats per group).

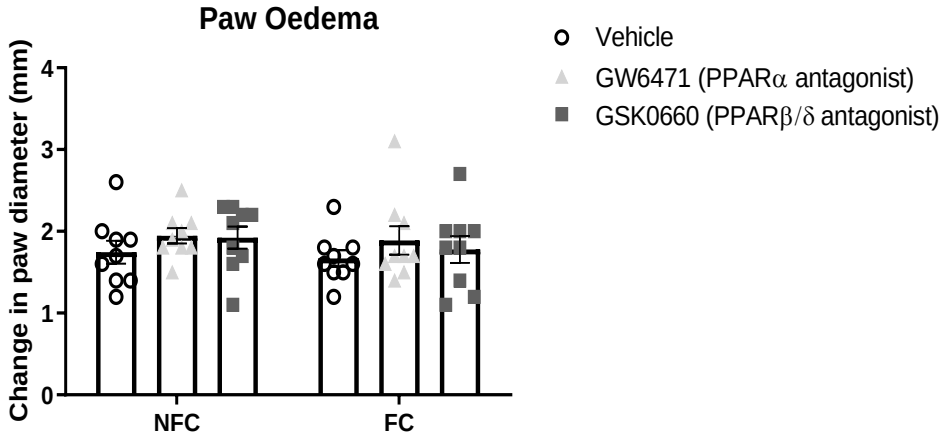


Figure 2.7: Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on formalin-evoked hind paw oedema in non-fear conditioned (NFC) and fear conditioned (FC) rats. Data are expressed as mean ± S.E.M, n=9 rats per group.

2.3.1.2 Effects of systemic administration of GW6471 and GSK0660 on fear-related behaviour in formalin-treated rats

The analysis of the duration of freezing (Figure 2.8) with two-way repeated-measures ANOVA revealed a significant effect of time [F (2.871, 132.072) = 7.213, $p<0.001$], conditioning [F (1, 46) = 80.397, $^{a}p<0.001$], time x conditioning [F (2.871, 132.072) = 5.961, p=0.001] but not of time x treatment [F (5.742, 132.072) = 1.455, $p>0.05$], treatment [F (1, 46) = 0.202, $p>0.05$], conditioning x treatment [F (2, 46) = 1.803, $p>0.05$] and time x conditioning x treatment [F (5.742, 132.072) = 1.584, $p>0.05$]. *Post hoc* analysis with Student Newman-Keuls confirmed that vehicle and drug-treated FC rats had significantly greater levels of freezing than NFC counterparts at all time bins. *Post hoc* analysis also revealed that treatment with GW6471 in FC rats significantly increased freezing duration in two of the 3-min time bins: 7-9 and 10-12 ($^{\#}p<0.05$), compared with vehicle-treated FC counterparts. In addition, treatment with GSK0660 significantly increased freezing duration in one of the 3-min time bins: 10-12 ($^{\$}p<0.05$), compared with vehicle-treated FC counterparts.

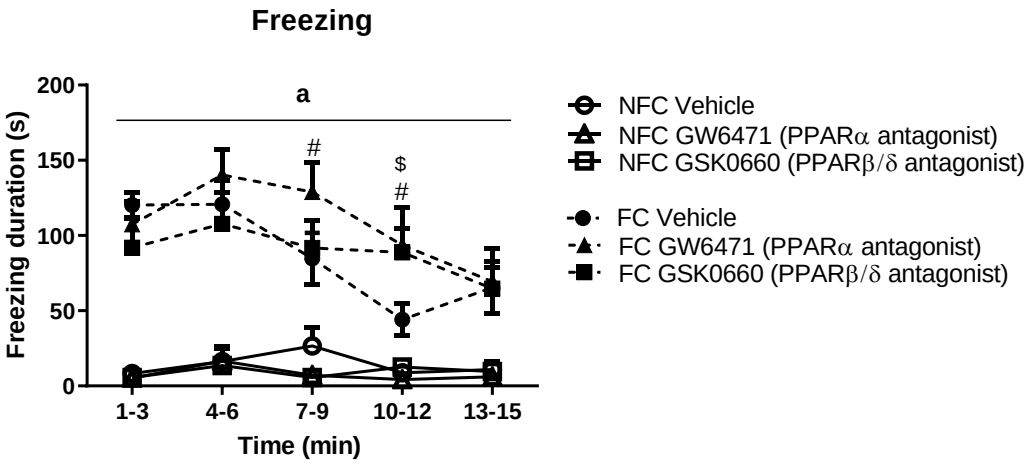


Figure 2.8: Temporal profile of the effects of fear conditioning and systemic administration of selective PPARα and PPARβ/δ antagonists on freezing in non-fear conditioned (NFC) and fear conditioned (FC) rats. *Post hoc* analysis with Student Newman-Keuls revealed that all formalin-injected FC groups exhibited significantly greater duration of freezing compared with NFC counterparts ($^{a}p<0.001$). Treatment with GW6471 in FC rats significantly increased freezing duration in two of the 3-min time bins ($^{\#}p<0.05$, vs FC Vehicle), and treatment with GSK0660 significantly increased freezing duration in one of the 3-min time bins ($^{\$}p<0.05$, vs FC Vehicle). Data are expressed as mean ± S.E.M (n=7-9 per group).

The analysis of the number of faecal pellets excreted (defecation; Figure 2.9) with Kruskal Wallis test revealed a significant difference among groups (χ^2 (5) = 38.90, p<0.001). *Post hoc* analysis with Dunn's test revealed that the number of faecal pellets excreted was significantly higher in all FC rats when compared to all their NFC counterparts (FC Vehicle vs NFC Vehicle groups [**p=0.0016], FC GW6471 vs NFC GW6471 [$^{\#\#}$p=0.0044], and FC GSK0660 vs NFC GSK0660 [$^{\$}$p<0.05]). Neither GW6471 nor GSK0660 had any significant effect on defecation in non-fear-conditioned or fear-conditioned rats.

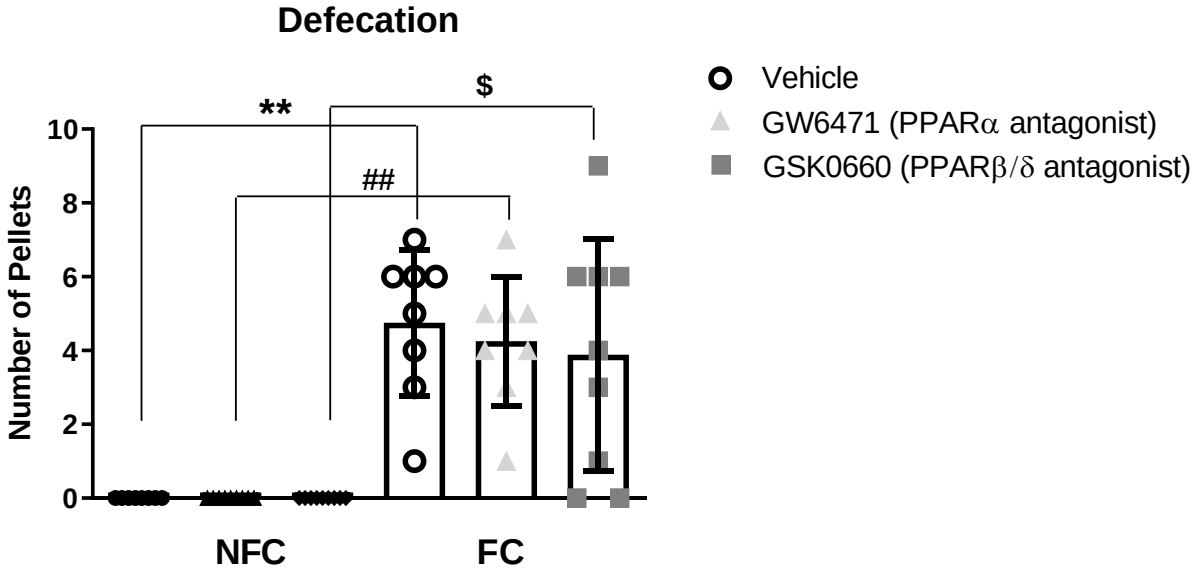


Figure 2.9: Effects of systemic administration of selective PPARα and PARβ/δ antagonists on defecation in non-fear conditioned (NFC) and fear conditioned (FC) rats. Post hoc with Dunn's test revealed that all formalin-injected FC groups exhibited significantly increased number of excreted faecal pellets compared with NFC counterparts (**p<0.01, vs NFC Vehicle; $^{\#\#}$p<0.01, vs NFC GW6471; $^{\$}$p<0.05, vs NFC GSK0660). Data are expressed as median with interquartile range and min/max (n=9 rats per group).

2.3.1.3 Effects of systemic administration of GW6471 and GSK0660 on general/motor behaviour

The analysis of walking duration (Figure 2.10A) with repeated measures two-way ANOVA revealed a significant effect of fear conditioning [F (1, 48) = 110.009, ^{a}p<0.001], time [F (3.031, 145.511) = 23.695, p<0.05], time x fear conditioning [F (3.031, 145.511) = 22.537, p<0.05], but not of treatment [F (2, 48) = 0.284, p>0.05], treatment x fear

conditioning [F (2, 48) = 1.202, p>0.05], treatment x time [F (6.063, 145.511) = 0.794, p>0.05], treatment x fear conditioning x time [F (6.063, 145.511) = 0.393, p>0.05] . *Post hoc* analysis with Student Newman-Keuls test indicated that walking duration was significantly lower all FC groups compared to their NFC counterparts in four time bins [FC Vehicle vs NFC Vehicle (Time $_{1\text{-}3}$, $p<0.01$; Time $_{4\text{-}6}$, $p<0.01$; Time $_{7\text{-}9}$, $p<0.001$; Time $_{10\text{-}12}$, $p<0.01$;), FC GW6471 vs NFC GW6471 (Time $_{1\text{-}3}$, $p<0.01$; Time $_{4\text{-}6}$, $p<0.01$; Time $_{7\text{-}9}$, $p<0.001$; Time $_{10\text{-}12}$, $p<0.001$), and FC GSK0660 vs NFC GSK0660 (Time $_{1\text{-}3}$, $p<0.05$; Time $_{4\text{-}6}$, $p<0.01$; Time $_{7\text{-}9}$, $p<0.01$; Time $_{10\text{-}12}$, $p<0.05$)]. Neither GW6471 nor GSK0660 had any significant effect on walking in non-fear-conditioned or fear-conditioned rats.

The analysis of grooming duration (Figure 2.10B) with Friedman's test did not show any significant effect of time (χ^2 (4) = 3.388, p>0.05) . Kruskal Wallis test revealed a significant difference among groups in two of the time bins [($\chi^2_{1\text{-}3}$ (5) = 31.635, $p<0.001$; $\chi^2_{13\text{-}15}$ (5) = 12.510, $p<0.05$)]. *Post hoc* analysis with Dunn's test indicated that grooming was significantly lower in the FC GW6471 group compared to NFC GW6471 in the first time bin (Time $_{1\text{-}3}$, $^{\#\#}p<0.01$) and in the FC GSK0660 group compared to NFC GSK0660 in the last time bin (Time $_{13\text{-}15}$, $^{\$}p<0.05$).

The analysis of rearing duration (Figure 2.10C) with Friedman's test revealed a significant effect of time (χ^2 (4) = 22.234, $p<0.001$) . *Post hoc* analysis with Wilcoxon's test indicated that rearing was significantly lower at $Time_{4\text{-}6}$ compared to $Time_{1\text{-}3}$ ($p=0.001$), $Time_{13\text{-}15}$ compared to $Time_{1\text{-}3}$ ($p<0.01$), and $Time_{7\text{-}9}$ compared to $Time_{13\text{-}15}$ ($p<0.05$). Kruskal Wallis test revealed a significant difference among groups in one of the time bins [($\chi^2_{10\text{-}12}$ (5) = 11.987, $p<0.05$]. *Post hoc* analysis with Dunn's test did not indicate significant differences between groups in rearing duration in that time bin.

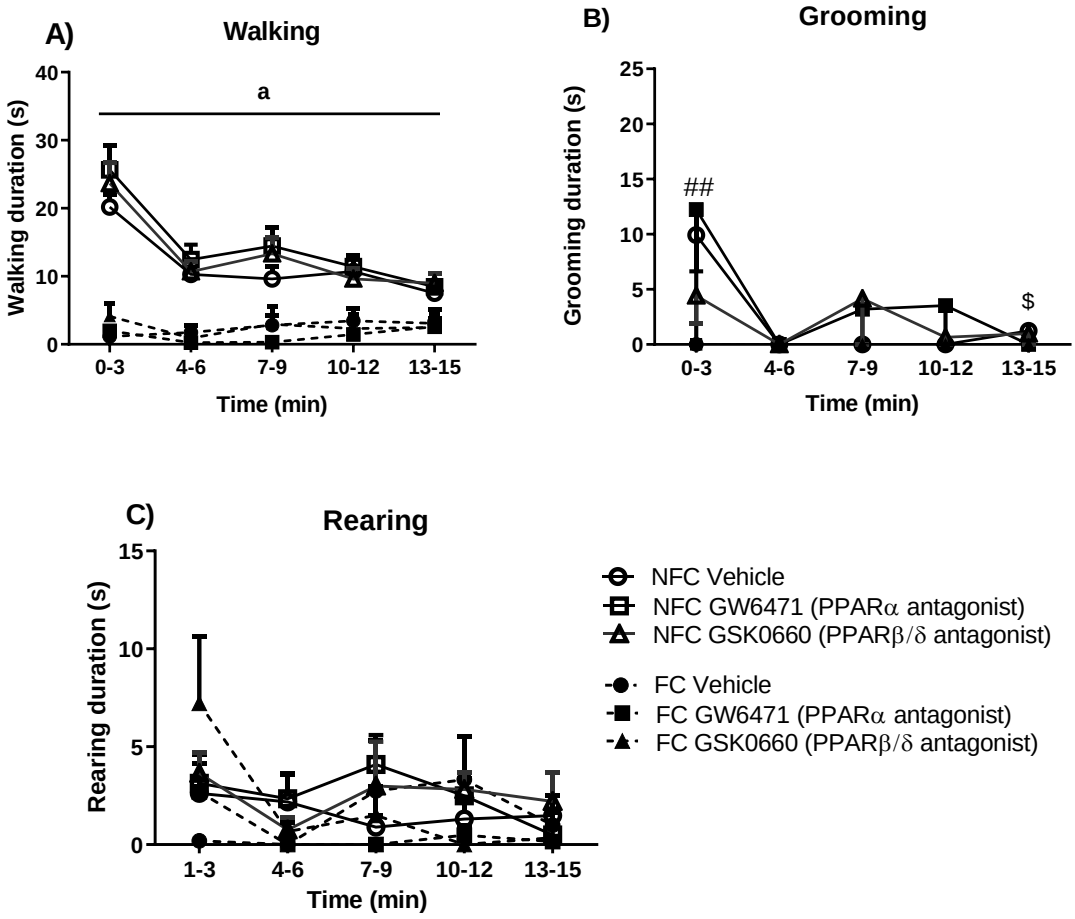


Figure 2.10: Temporal profile of the effects of fear-conditioning and systemic administration of selective PPARα and PPARβ/δ antagonists on walking duration (A), grooming duration (B), and rearing duration (C). Post hoc analysis with Student Newman-Keuls test indicated that walking duration (A) was significantly lower all formalin-injected FC groups ($^{a}p<0.05$, vs FC-counterpart). Post hoc analysis with Dunn's test indicated that grooming (B) was significantly lower in the FC GW6471 group compared to NFC GW6471 in the first time bin ($^{\#\#}p<0.01$) and in the FC GSK0660 group compared to NFC GSK0660 in the last time bin ($^{\$}p<0.05$). Data are expressed as mean ± S.E.M (A) or median with interquartile range and min/max (B and C) (n=9 rats per group).

2.3.1.4 Effect of fear conditioning and GW6471 and GSK0660 administration on neurotransmitter levels in the BLA, CeA, and VH of formalin-treated rats

The PPAR signalling system has previously been shown to be associated with positive changes in the GABAergic (Sasso et al., 2010), promotes an increase in the expression of glutamatergic receptors (Ching et al., 2015), and increased levels of serotonin

(Waku et al., 2010; Mijangos-Moreno et al., 2016) and dopamine (Mascia et al., 2011; Mijangos-Moreno et al., 2016b; Chikahisa et al., 2019). Therefore, the blockade of PPARs could affect levels of one or more of these neurotransmitters, which it turn play key roles in pain and fear. In order to check if the alterations in pain and/or fear responses after fear conditioning and treatment with PPAR antagonists were associated with changes in the levels of neurotransmitters, we examined tissue levels of GABA, glutamate, serotonin and dopamine in the BLA, CeA, and VH.

2.3.1.4.1 Effect of fear conditioning and GW6471 and GSK0660 administration on neurotransmitter levels in the BLA

Levels of GABA analysed using three-way ANOVA revealed an overall treatment effect [$F(2, 76) = 5.628$, $^{a}p<0.01$] (Figure 2.11A). However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. Fear conditioning [$F(1, 76) = 0.054$, $p>0.05$] and side [$F(1, 76) = 2.669$, $p>0.05$] did not affect levels of GABA in the BLA. The interaction of treatment x conditioning [$F(2, 76) = 1.133$, $p>.05$], treatment x side [$F(2, 76) = 0.017$, $p>0.05$], conditioning x side [$F(1, 76) = 0.237$, $p>0.05$], and treatment x conditioning x side [$F(2, 76) = 0.648$, $p>0.05$] did not significantly affect GABA levels in the BLA. When each side was analysed separately, two-way ANOVA revealed an effect of treatment on GABA levels in the right side [$F(2, 44) = 3.910$, $p<0.05$] but not in the left side [$F(2, 37) = 2.172$, $p>0.05$]. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. Fear conditioning [Right $F(1, 44) = 0.546$, $p>0.05$; Left $F(1, 37) = 0.621$, $p>0.05$] and treatment x fear conditioning [Right $F(2, 44) = 0.075$, $p>0.05$; Left $F(2, 37) = 2.005$, $p>0.05$] did not affect GABA levels in the BLA.

Levels of glutamate analysed using three-way ANOVA revealed that treatment [$F(2, 76) = 2.307$, $p>0.05$], fear conditioning [$F(1, 76) = 0.007$, $p>0.05$] and side [$F(1, 76) = 0.264$, $p>0.05$] did not have any effect on its levels in the BLA (Figure 2.11B). The interaction of treatment x conditioning [$F(2, 76) = 0.069$, $p>0.05$], treatment x side [$F(2, 76) = 0.439$, $p>0.05$], conditioning x side [$F(1, 76) = 0.658$, $p<0.05$], and treatment x conditioning x side [$F(2, 76) = 1.394$, $p>0.05$] did not significantly affect glutamate levels in the BLA. When each side was analysed separately, two-way ANOVA revealed no effect of treatment on glutamate levels either in the right side [$F(2, 39) = 0.310$, $p>0.05$] or in the left side [$F(2, 37) = 3.037$, $p>0.05$]. Fear conditioning [Right $F(1, 39) = 0.220$, $p>0.05$; Left

F (1, 37) = 0.514, p>0.05] and treatment x fear conditioning [Right F (2, 39) = 0.386, p>0.05; Left F (2, 37) = 1.247, p>0.05] did not affect glutamate levels in the BLA.

Levels of serotonin analysed using three-way ANOVA revealed an overall conditioning effect [F (2, 85) = 3.975, p<0.05] (Figure 2.11C). However, *post hoc* pairwise group comparisons did not reach statistical significance. Treatment [F (2, 85) = 0.368, p>0.05] and side [F (1, 85) = 0.634, p>0.05] did not affect levels of serotonin in the BLA. The interaction of treatment x conditioning [F (2, 85) = 0.438, p>0.05], treatment x side [F (2, 85) = 0.253, p>0.05], conditioning x side [F (1, 85) = 0.206, p>0.05], and treatment x conditioning x side [F (2, 85) = 0.382, p>0.05] did not significantly affect serotonin levels in the BLA. When each side was analysed separately, two-way ANOVA revealed no effect of treatment on serotonin levels either in the right side [F (2, 44) = 0.280, p>0.05] or in the left side [F (2, 41) = 0.489, p>0.05]. Fear conditioning [Right F (1, 44) = 0.700, p>0.05; Left F (1, 41) = 2.412, p>0.05] and treatment x fear conditioning [Right F (2, 44) = 0.305, p>0.05; Left F (2, 41) = 0.004, p>0.05] did not affect serotonin levels in the BLA.

Levels of dopamine analysed using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 5.742, p>0.05] in the BLA (Figure 2.11D). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 3.389, p>0.05; Left χ^2 (5) = 1.922, p>0.05].

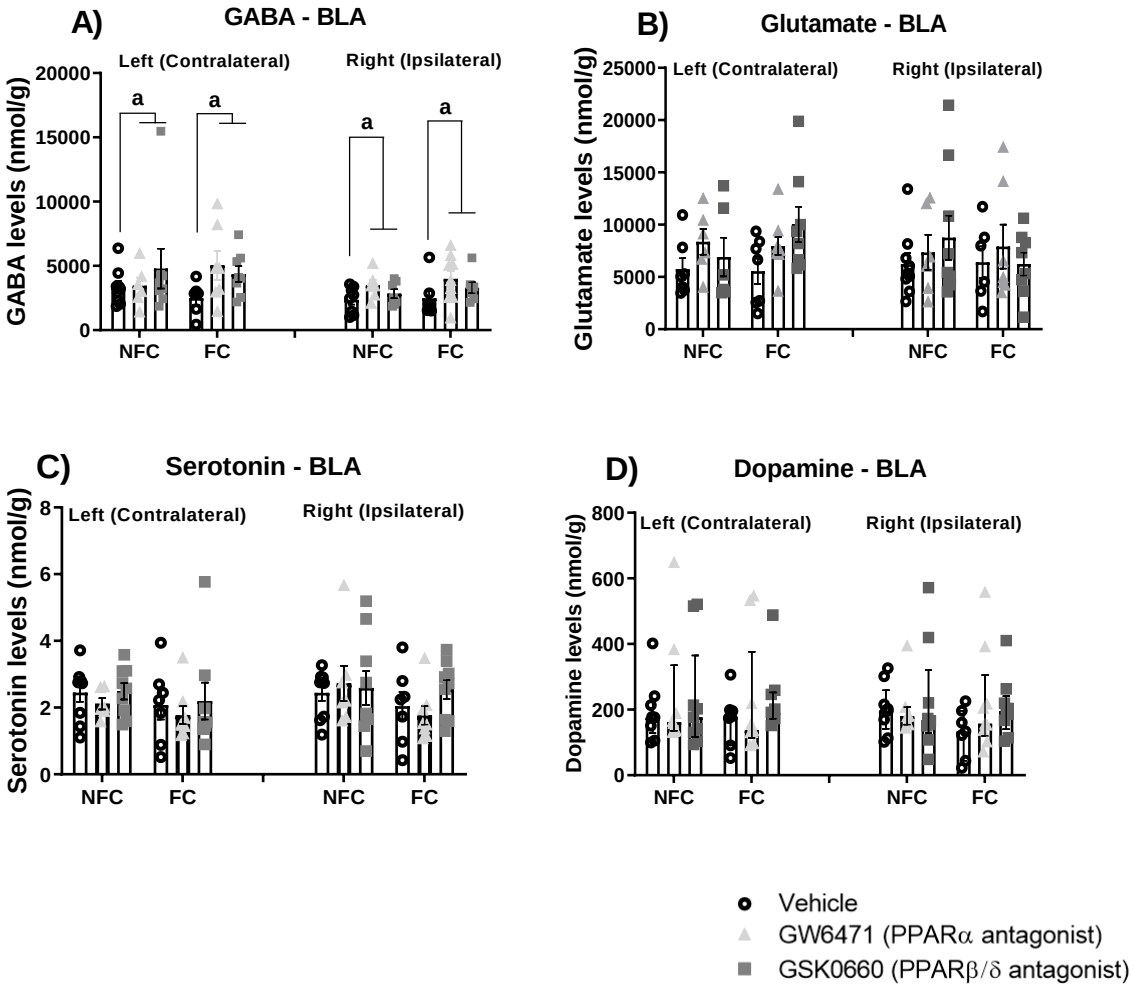


Figure 2.11*:* Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on GABA (A), glutamate (B), serotonin (C), and dopamine (D) levels in the basolateral amygdala (BLA) in formalin-injected non-fear conditioned (NFC) and fear conditioned (FC) rats. Three-way ANOVA revealed an overall treatment effect on levels of GABA ($^{a}p<0.01$). Data are expressed as mean ± S.E.M (A, B, and C) or median with interquartile range and min/max (D) (n=7-9 rats per group).

2.3.1.4.2 Effect of fear conditioning and GW6471 and GSK0660 administration on neurotransmitter levels in the central nucleus of the amygdala (CeA)

The analysis of the levels of GABA using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 10.018, p>0.05] in the CeA (Figure 2.12A). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 4.630, p>0.05; Left χ^2 (5) = 4.041, p>0.05].

The analysis of the levels of glutamate using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 8.882, p>0.05] in the CeA (Figure 2.12B). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 7.868, p>0.05; Left χ^2 (5) = 1.067, p>0.05].

The analysis of the levels of serotonin using Kruskal Wallis test revealed a significance difference among groups [χ^2 (11) = 20.669, p<0.05] in the CeA (Figure 2.12C). However, *post hoc* pairwise group comparisons with Dunn's test did not reach statistical significance. The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 0.783, p>0.05; Left χ^2 (5) = 5.368, p>0.05].

The analysis of the levels of dopamine using three-way ANOVA revealed an overall treatment [F (2, 80) = 3.181, p=0.047], side [F (1, 80) = 35.257, p<0.001], and treatment x conditioning [F (2, 80) = 3.994, p<0.022] (Figure 2.12D). However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. Fear conditioning [F (1, 80) = 2.430, p>0.05] did not affect levels of dopamine in the CeA. The interaction of treatment x side [F (2, 80) = 0.146, p>0.05], conditioning x side [F (1, 80) = 0.082, p>0.05], and treatment x conditioning x side [F (2, 80) = 0.234, p>0.05] did not significantly affect dopamine levels in the CeA. When each side was analysed separately, two-way ANOVA revealed no effect of treatment either on dopamine levels in the right side [F (2, 39) = 1.967, p>0.05] or in the left side [F (2, 41) = 0.936, p>0.05]. Fear conditioning [Right F (1, 39) = 0.737, p>0.05; Left F (1, 41) = 1.488, p>0.05] and treatment x fear conditioning [Right F (2, 39) = 3.184, p=0.052; Left F (2, 41) = 1.550, p>0.05] did not affect dopamine levels in the CeA. Due to the trend seen in the interaction of treatment x fear conditioning in right side (p=0.052, see above), I compared each conditioning group in each side separately. In this scenario, one-way ANOVA reveals a significant effect of treatment on dopamine levels in the right CeA of FC [F (2, 23) = 3.868, $^{\$}$p=0.043] but not in NFC [F

(2, 23) = 2.106, p>0.05] rats. *Post doc* analysis with Student Newman-Keuls test indicates that dopamine is significantly higher in FC GW6471-treated compared to FC Vehicle-treated rats ($^{\#}p<0.05$). Treatment did not have an effect on dopamine levels in the left side neither in FC [F (2, 21) = 0.341, p>0.05] nor NFC rats [F (2, 20) = 2.107, p>0.05].

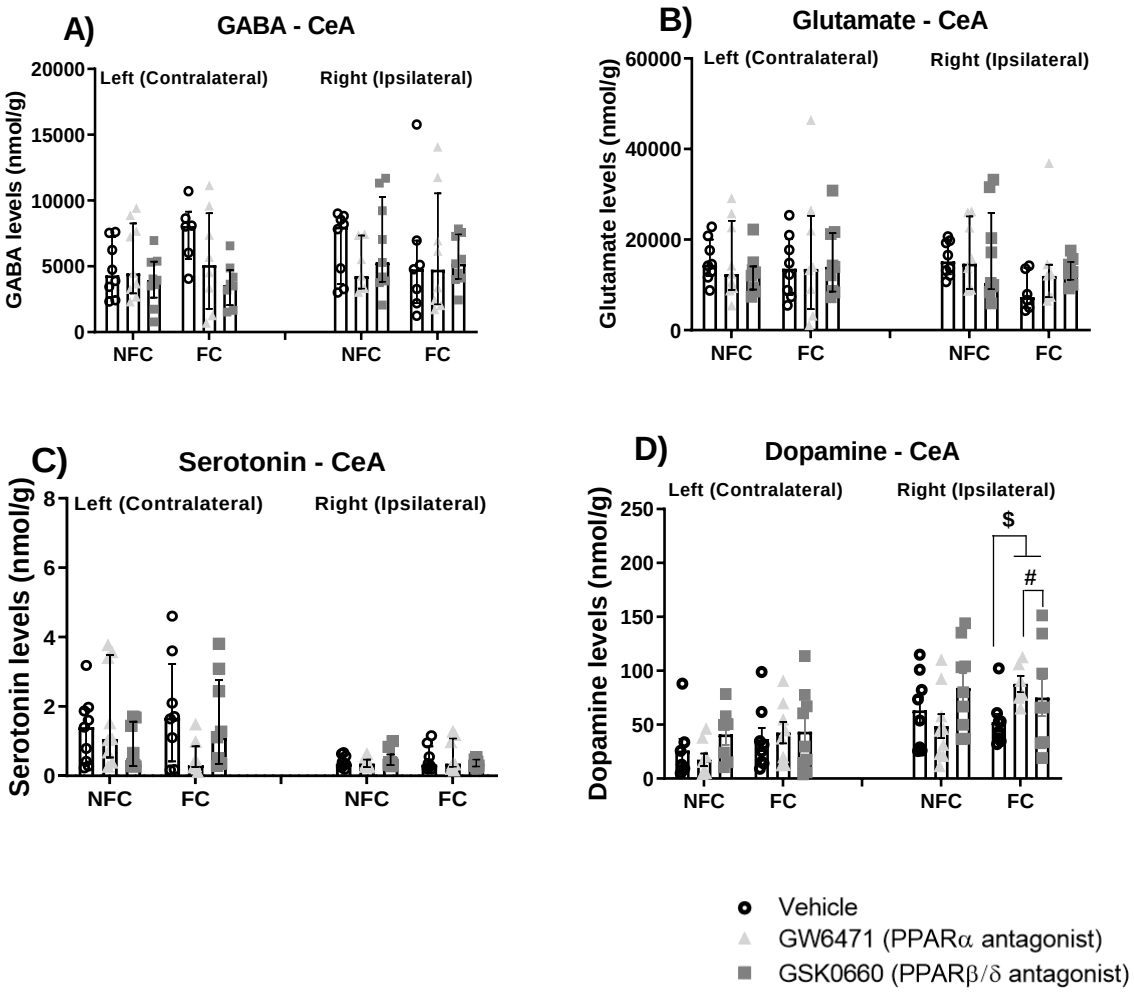


Figure 2.12: Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on GABA (A), glutamate (B), serotonin (C), and dopamine (D) levels in the central nucleus of the amygdala (CeA) in formalin-injected non-fear conditioned (NFC) and fear conditioned (FC) rats. One-way ANOVA reveals a significant effect of treatment on dopamine levels in the right CeA of FC ($^{\$}p=0.043$) rats. Post doc analysis indicated that dopamine is significantly higher in FC GW6471-treated compared to FC Vehicle-treated rats ($^{\#}p<0.05$). Data are expressed as mean ± S.E.M (D) and median with interquartile range (A, B and C), n=7-9 rats per group.

There was no significant correlation between the level of dopamine in the right CeA of FC rats treated with vehicle and the duration of freezing [r = 0.08513; p>0.05; R^2 = 0.007] (Figure 2.13A). There was no significant correlation between the level of dopamine in the right CeA of FC rats treated with GW6471 and the duration of freezing [r = 0.1069; p>0.05; R^2 = 0.01144] Figure 2.13B).

There was a significant positive correlation between the level of dopamine in the right CeA of FC rats treated with GSK0660 and the duration of freezing [r = -0.8201; p=0.0127; R^2 = 0.6725] Figure 2.13C).

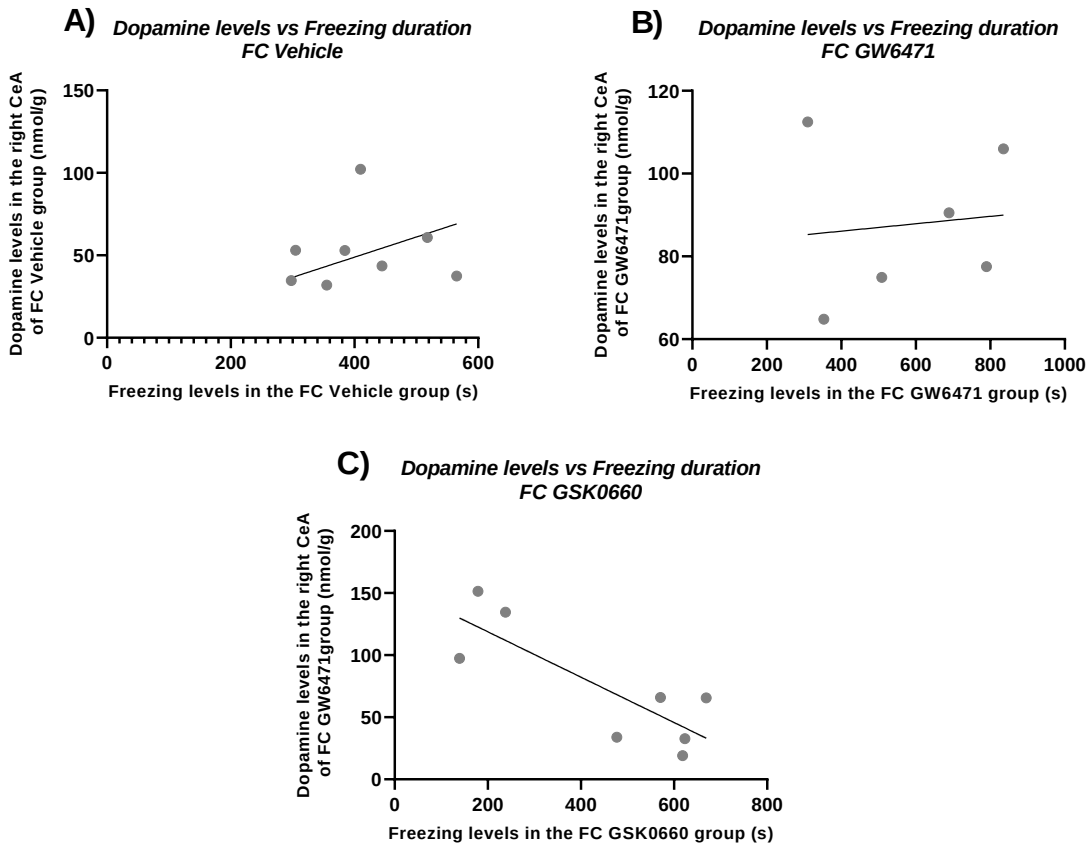


Figure 2.13: Correlation between dopamine levels in the CeA of FC rats treated with vehicle (A), GW6471 (B), and GSK0660 (C) and freezing duration.

2.3.1.4.3 Effect of fear conditioning and GW6471 and GSK0660 administration on neurotransmitter levels in the ventral hippocampus (VH)

The analysis of the levels of GABA using three-way ANOVA revealed treatment [F (2, 88) = 3.193, p<0.05] and side [F (1, 88) = 27.473, p<0.001] effects (Figure 2.14A).

However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. Fear conditioning [$F(1, 88) = 0.575$, $p>0.05$] did not affect levels of GABA in the VH. The interaction of treatment x side [$F(2, 88) = 0.577$, $p>0.05$], conditioning x side [$F(1, 88) = 0.229$, $p>0.05$], treatment x conditioning [$F(2, 88) = 0.577$, $p>0.05$] and treatment x conditioning x side [$F(2, 88) = 1.269$, $p>0.05$] did not significantly affect GABA levels in the VH. When each side was analysed separately, two-way ANOVA revealed no effect of treatment on GABA levels either in the right side [$F(2, 44) = 2.205$, $p>0.05$] or in the left side [$F(2, 44) = 1.569$, $p>0.05$]. Fear conditioning [Right $F(1, 44) = 0.210$, $p>0.05$; Left $F(1, 44) = 0.142$, $p>0.05$] and treatment x fear conditioning [Right $F(2, 44) = 2.004$, $p>0.05$; Left $F(2, 44) = 0.029$, $p>0.05$] did not affect GABA levels in the VH.

The analysis of the levels of glutamate using Kruskal Wallis test revealed a significance difference among groups [$\chi^2(11) = 27.578$, $p<0.05$] in the VH (Figure 2.14B). However, *post hoc* pairwise group comparisons with Dunn's test did not reach statistical significance. The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right $\chi^2(5) = 9.537$, $p>0.05$; Left $\chi^2(5) = 3.816$, $p>0.05$].

The analysis of the levels of serotonin using three-way ANOVA revealed a side [$F(1, 92) = 43.602$, $p<0.001$] and treatment [$F(2, 92) = 3.764$, $p=0.027$] effect (Figure 2.14C). Student Newman-Keuls *post hoc* pairwise group comparisons indicated that levels of serotonin were significantly lower on the right side of the VH of both vehicle groups compared to the left side (Right NFC Vehicle vs Left NFC Vehicle, $^*p<0.05$; Right FC Vehicle vs Left FC Vehicle, $^*p<0.05$) and also for NFC GW6471-treated animals (Right NFC GW6471 vs Left NFC GW6471, $^*p<0.05$). Fear conditioning [$F(1, 92) = 1.459$, $p>0.05$] did not affect levels of serotonin in the VH. The interaction of treatment x side [$F(2, 92) = 0.282$, $p>0.05$], conditioning x side [$F(1, 92) = 0.174$, $p>0.05$], treatment x conditioning [$F(2, 92) = 0.293$, $p>0.05$] and treatment x conditioning x side [$F(2, 92) = 0.092$, $p>0.05$] did not significantly affect serotonin levels in the VH. When each side was analysed separately, two-way ANOVA revealed no effect of treatment on serotonin levels either in the right side [$F(2, 45) = 2.588$, $p>0.05$] or in the left side [$F(2, 47) = 1.255$, $p>0.05$]. Fear conditioning [Right $F(1, 45) = 1.190$, $p>0.05$; Left $F(1, 47) = 0.348$, $p>0.05$] and treatment x fear conditioning [Right $F(2, 45) = 0.322$, $p>0.05$; Left $F(2, 47) = 0.039$, $p>0.05$] did not affect serotonin levels in the VH.

The analysis of the levels of dopamine using three-way ANOVA revealed a side [$F(1, 89) = 53.723$, $p<0.001$] effect (Figure 2.14D). Student Newman-Keuls *post hoc* pairwise group comparisons indicated that levels of dopamine were significantly lower on the right side of the VH of the FC vehicle group compared to the left side (Right FC Vehicle vs Left FC Vehicle, $*p<0.05$) and also for NFC GSK0660-treated animals (Right NFC GSK0660 vs Left NFC GSK0660, $*p<0.05$). Fear conditioning [$F(1, 89) = 1.041$, $p>0.05$] and treatment [$F(2, 89) = 1.651$, $p>0.05$] did not affect levels of dopamine in the VH. The interaction of treatment x side [$F(2, 89) = 0.731$, $p>0.05$], conditioning x side [$F(1, 89) = 0.623$, $p>0.05$], treatment x conditioning [$F(2, 89) = 0.302$, $p>0.05$] and treatment x conditioning x side [$F(2, 89) = 1.142$, $p>0.05$] did not significantly affect dopamine levels in the VH. When each side was analysed separately, two-way ANOVA revealed no effect of treatment on dopamine levels either in the right side [$F(2, 43) = 1.851$, $p>0.05$] or in the left side [$F(2, 46) = 0.897$, $p>0.05$]. Fear conditioning [Right $F(1, 43) = 0.022$, $p>0.05$; Left $F(1, 46) = 2.139$, $p>0.05$] and treatment x fear conditioning [Right $F(2, 43) = 1.590$, $p>0.05$; Left $F(2, 46) = 0.129$, $p>0.05$] did not affect dopamine levels in the VH.

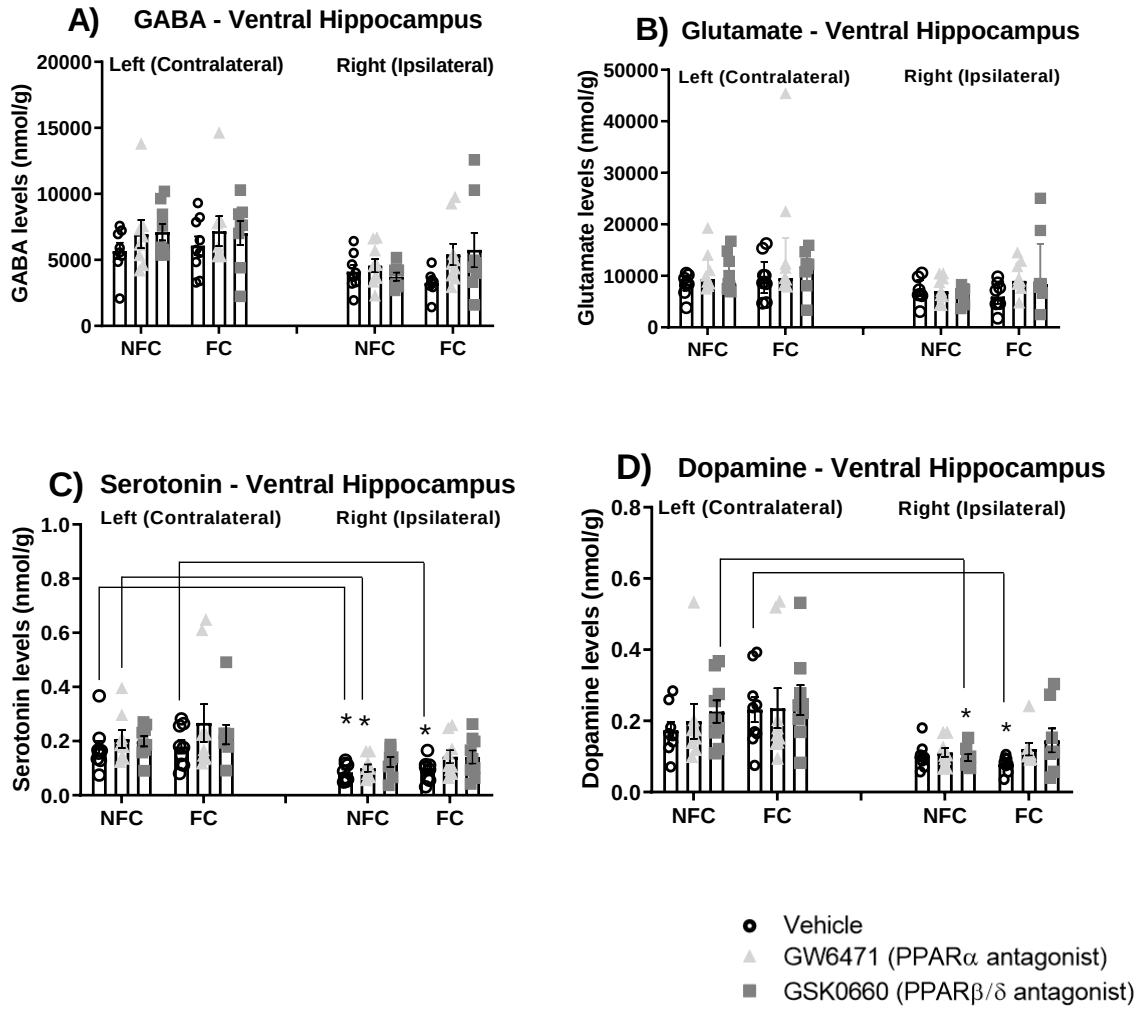


Figure 2.134: Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on GABA (A), glutamate (B), serotonin (C), and dopamine (D) levels in the ventral hippocampus (VH) in formalin-injected non-fear conditioned (NFC) and fear conditioned (FC) rats. Post hoc test indicated that levels of serotonin and dopamine were significantly lower on the right side of the VH (Right vs Left, *$p<0.05$). Data are expressed as mean ± S.E.M (A, C and D) or median with interquartile range (B), n=7-9 rats per group.

2.3.1.5 Effect of fear conditioning and GW6471 and GSK0660 administration on endocannabinoid and NAE levels in the BLA, CeA, and VH

In order to check if the alterations in pain and/or fear responses after fear conditioning and treatment with PPAR antagonists were associated with changes in the levels of the endogenous ligands (i.e. NAEs) and endocannabinoids, we examined tissue levels of 2-AG, AEA, PEA and OEA in the BLA, CeA, and VH.

2.3.1.5.1 Effect of fear conditioning and GW6471 and GSK0660 administration on endocannabinoid and NAE levels in the BLA

The analysis of the levels of 2-AG using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 14.299, p>0.05] in the BLA (Figure 2.15A). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 5.188, p>0.05; Left χ^2 (5) = 8.578, p>0.05].

The analysis of the levels of AEA using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 12.565, p>0.05] in the BLA (Figure 2.15B). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 3.349, p>0.05; Left χ^2 (5) = 2.653, p>0.05].

The analysis of the levels of PEA using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 6.761, p>0.05] in the BLA (Figure 2.15C). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 0.970, p>0.05; Left χ^2 (5) = 5.305, p>0.05].

The analysis of the levels of OEA using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 6.688, p>0.05] in the BLA (Figure 2.15D). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 1.586, p>0.05; Left χ^2 (5) = 4.983, p>0.05].

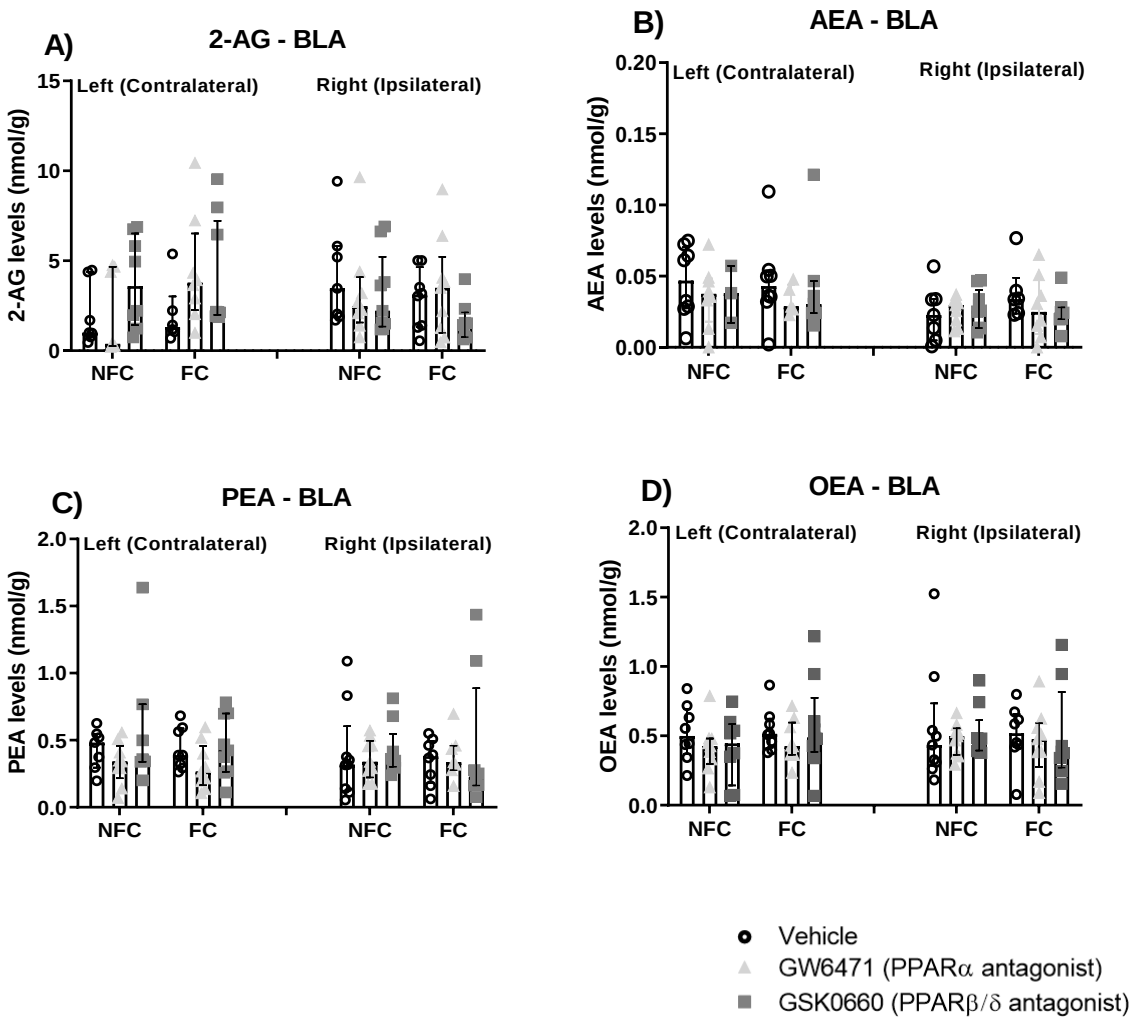


Figure 2.145: Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on 2-AG (A), AEA (B), PEA (C), and OEA (D) levels in the basolateral amygdala (BLA) in non-fear conditioned (NFC) and fear conditioned (FC) rats. Data are expressed as median with interquartile range (n=7-9 rats per group).

2.3.1.5.2 Effect of fear conditioning and GW6471 and GSK0660 administration on endocannabinoid and NAEs levels in the CeA

The analysis of the levels of 2-AG using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 14.405, p>0.05] in the CeA (Figure 2.16A). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 2.926, p>0.05; Left χ^2 (5) = 10.556, p>0.05].

The analysis of the levels of AEA using Kruskal Wallis test did not show any significant difference among groups [χ^2 (11) = 9.064, p>0.05] in the CeA (Figure 2.16B). The analysis of each of the sides separately by Kruskal Wallis did not reveal any significant difference among groups [Right χ^2 (5) = 4.859, p>0.05; Left χ^2 (5) = 3.234, p>0.05].

The analysis of the levels of PEA using three-way ANOVA revealed that side [F (1, 89) = 1.508, p>0.05], fear conditioning [F (1, 89) = 1.723, p>0.05] and treatment [F (2, 89) = 0.455, p>0.05] did not affect levels of PEA in the CeA (Figure 2.16C). There was a significant interaction of treatment x side [F (2, 89) = 4.036, p=0.021]. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. The interaction of conditioning x side [F (1, 89) = 1.547, p>0.05], treatment x conditioning [F (2, 89) = 1.165, p>0.05] and treatment x conditioning x side [F (2, 89) = 0.694, p>0.05] did not significantly affect PEA levels in the CeA. When each side was analysed separately, two-way ANOVA revealed an effect of treatment on PEA levels in the right side [F (2, 44) = 3.403, p<0.05] but not in the left side [F (2, 45) = 0.981, p>0.05]. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. Fear conditioning [Right F (1, 44) = 3.126, p>0.05; Left F (1, 45) = 0.002, p>0.05] and treatment x fear conditioning [Right F (2, 44) = 0.441, p>0.05; Left F (2, 45) = 1.471, p>0.05].

The analysis of the levels of OEA using three-way ANOVA revealed an overall effect of side [F (1, 90) = 7.572, p=0.007]. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance (Figure 2.16D). Fear conditioning [F (1, 90) = 0.059, p>0.05] and treatment [F (2, 90) = 0.245, p>0.05] did not affect levels of OEA in the CeA (Figure 2.11 D). The interaction of treatment x side [F (2, 90) = 0.691, p>0.05], conditioning x side [F (1, 90) = 0.002, p>0.05], treatment x conditioning [F (2, 90) = 1.512, p>0.05] and treatment x conditioning x side [F (2, 90) = 0.936, p>0.05] did not significantly affect OEA levels in the CeA. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (2, 46) = 0.348, p>0.05; Left F (2, 44) = 0.596, p>0.05], fear conditioning [Right F (1, 46) = 0.019, p>0.05; Left F (1, 44) = 0.042, p>0.05], or treatment x fear conditioning [Right F (2, 46) = 1.240, p>0.05; Left F (2, 44) = 1.235, p>0.05] on OEA levels.

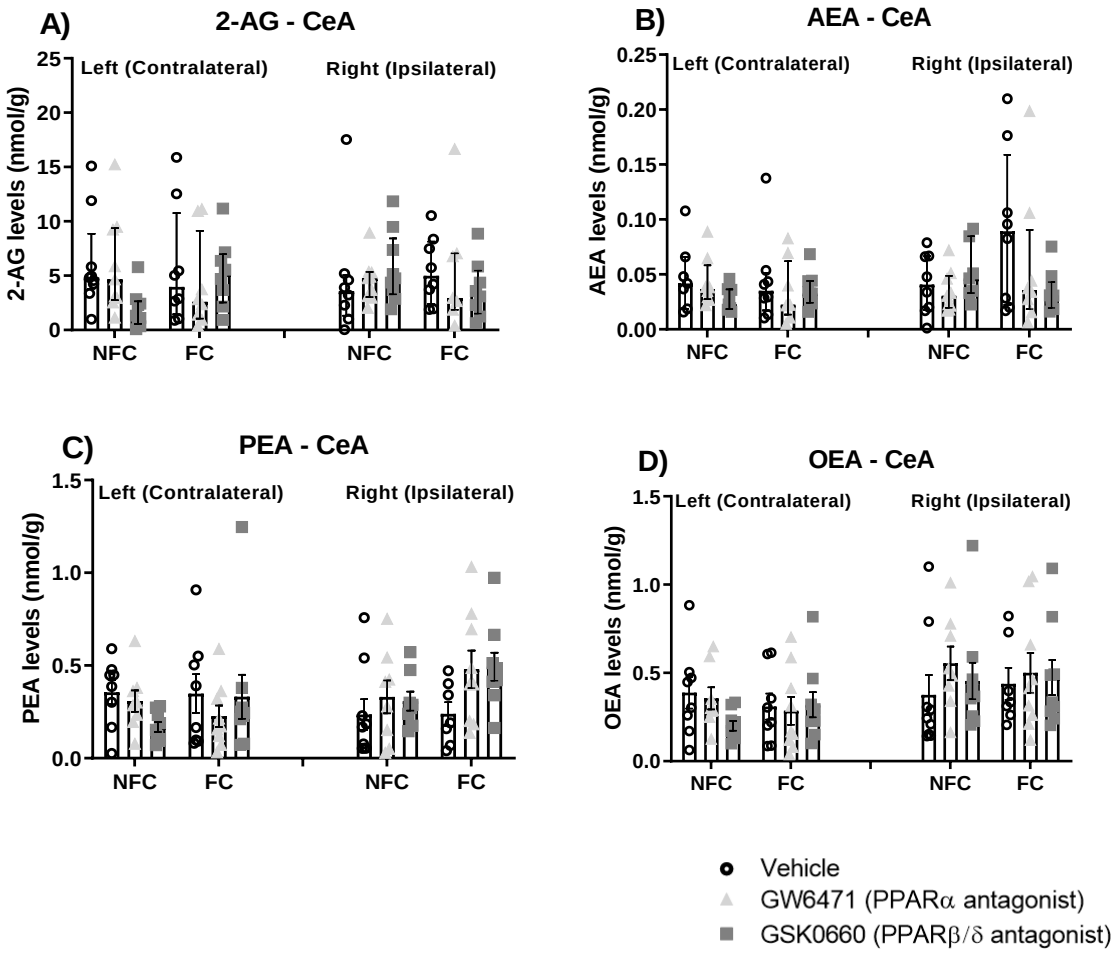


Figure 2.6: Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on 2-AG (A), AEA (B), PEA (C), and OEA (D) levels in the central nuclei of the amygdala (CeA) in non-fear conditioned (NFC) and fear conditioned (FC) rats. Data are expressed as median with interquartile range (A and B) and as mean ± S.E.M (C and D), n=7-9 rats per group.

2.3.1.5.3 Effect of fear conditioning and GW6471 and GSK0660 administration on endocannabinoid and NAEs levels in the VH

The analysis of the levels of 2-AG using three-way ANOVA revealed an overall effect of fear conditioning [F (1, 84) = 5.224, p=0.039] and side [F (1, 84) = 4.419, p=0.039] (Figure 2.17A). However, *post-hoc* pairwise group comparisons did not reach statistical significance. Treatment [F (2, 83) = 0.767, p>0.05] did not affect levels of 2-AG in the VH. The interaction of treatment x side [F (2, 83) = 2.648, p>0.05], conditioning x side [F (1, 83) = 0.872, p>0.05], treatment x conditioning [F (2, 83) = 0.645, p>0.05] and treatment x

conditioning x side [F (2, 83) = 0.169, p>0.05] did not significantly affect 2-AG levels in the VH. When each side was analysed separately, two-way ANOVA revealed an effect of treatment on 2-AG levels in the right side [F (2, 41) = 3.239, p<0.05] but not in the left side [F (2, 43) = 0.452, p>0.05] and of fear conditioning in the left side [F (1, 41) = 4.551, p<0.05] but not in the right side [F (1, 43) = 0.297, p>0.05]. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. The interaction of treatment x fear conditioning [Right F (2, 41) = 0.270, p>0.05; Left F (2, 43) = 0.543, p>0.05].

The analysis of the levels of AEA using three-way ANOVA revealed an overall effect of side [F (1, 90) = 8.292, p=0.005] (Figure 2.17B). However, *post hoc* pairwise group comparisons did not reach statistical significance. Fear conditioning [F (1, 90) = 0.275, p>0.05] and treatment [F (2, 90) = 0.310, p>0.05] did not affect levels of AEA in the VH. The interaction of treatment x side [F (2, 90) = 1.475, p>0.05], conditioning x side [F (1, 90) = 0.001, p>0.05], treatment x conditioning [F (2, 90) = 0.659, p>0.05] and treatment x conditioning x side [F (2, 90) = 0.200, p>0.05] did not significantly affect AEA levels in the VH. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (2, 44) = 1.450, p>0.05; Left F (2, 46) = 0.419, p>0.05], fear conditioning [Right F (1, 44) = 0.127, p>0.05; Left F (1, 46) = 0.149, p>0.05], or treatment x fear conditioning [Right F (2, 44) = 0.794, p>0.05; Left F (2, 46) = 0.095, p>0.05] on AEA levels.

The analysis of the levels of PEA using three-way ANOVA revealed that side [F (1, 88) = 0.867, p>0.05], fear conditioning [F (1, 88) = 0.001, p>0.05] and treatment [F (2, 88) = 0.255, p>0.05] did not have any effects on the levels of PEA in the VH (Figure 2.17C). The interaction of treatment x side [F (2, 88) = 1.940, p>0.05], conditioning x side [F (1, 88) = 0.008, p>0.05], treatment x conditioning [F (2, 88) = 0.346, p>0.05] and treatment x conditioning x side [F (2, 88) = 1.460, p>0.05] did not significantly affect PEA levels in the VH. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (2, 44) = 1.697, p>0.05; Left F (2, 44) = 0.491, p>0.05], fear conditioning [Right F (1, 44) = 0.133, p>0.05; Left F (1, 44) = 0.009, p>0.05], or treatment x fear conditioning [Right F (2, 44) = 0.931, p>0.05; Left F (2, 44) = 0.765, p>0.05] on PEA levels.

The analysis of the levels of OEA using three-way ANOVA revealed that side [F (1, 91) = 0.013, p>0.05], fear conditioning [F (1, 91) = 0.091, p>0.05] and treatment [F (2, 91) = 0.945, p>0.05] did not affect levels of OEA in the VH (Figure 2.17D). The interaction of treatment x side [F (2, 91) = 2.187, p>0.05], conditioning x side [F (1, 91) > 0.001, p>0.05], treatment x conditioning [F (2, 91) = 0.199, p>0.05] and treatment x conditioning x side [F (2, 91) = 0.900, p>0.05] did not significantly affect OEA levels in the VH. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (2, 45) = 2.369, p>0.05; Left F (2, 46) = 0.991, p>0.05], fear conditioning [Right F (1, 45) = 0.060, p>0.05; Left F (1, 46) = 0.036, p>0.05], or treatment x fear conditioning [Right F (2, 45) = 1.103, p>0.05; Left F (2, 46) = 0.163, p>0.05] on OEA levels.

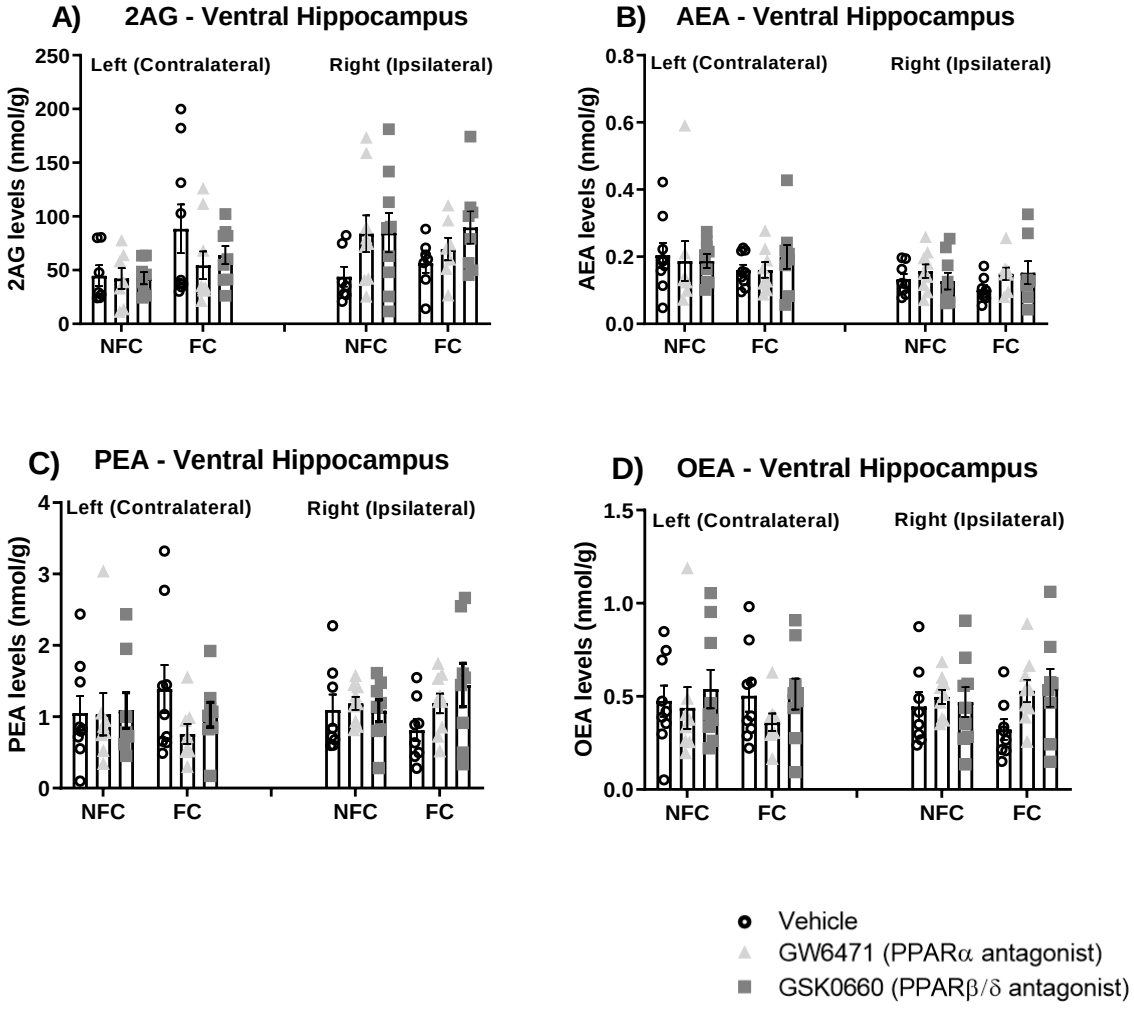


Figure 2.157: Effects of systemic administration of selective PPARα and PPARβ/δ antagonists on 2-AG (A), AEA (B), PEA (C), and OEA (D) levels in the ventral hippocampus (VH) in non-fear conditioned (NFC) and fear conditioned (FC) rats. Data are expressed as mean ± S.E.M, n=7-9 rats per group.

2.3.2 Experiment 2

2.3.2.1 Effects of systemic administration of GW9662 on formalin-evoked nociceptive behaviour and FCA

As in Experiment 1, intra-plantar administration of formalin into the right hind paw produced robust nociceptive behaviour as evidenced by the composite pain score. Kruskal-Wallis comparisons revealed a significant difference among all groups (χ^2 (3) = 27.226, $p<0.001$) in total composite pain score values (Figure 2.18A). *Post hoc* analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in both FC groups compared with their NFC counterparts, confirming expression of FCA (FC Vehicle vs NFC Vehicle [**$p<0.001$]; FC GW9662 vs NFC GW9662 [$^{+++}p<0.001$]). The treatment with GW9662 did not have any significant effect on formalin-evoked nociceptive behaviour in NFC or FC rats.

The analysis of pain 1 duration (see definition in the section 2.2.4) (Figure 2.18B) using Kruskal-Wallis comparisons revealed a significant difference among all groups (χ^2 (3) = 27.23, $p<0.001$). *Post hoc* analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in both fear-conditioned groups compared with their non-fear-conditioned counterparts (FC Vehicle vs NFC Vehicle [**$p<0.001$]; FC GW9662 vs NFC GW9662 [$^{+++}p<0.001$]). The treatment with GW9662 did not have any significant effect on pain 1 duration in NFC or FC rats.

The analysis of pain 2 duration (see definition in the section 2.2.4) (Figure 2.18C) using Kruskal-Wallis comparisons revealed a significant difference among all groups (χ^2 (3) = 30.18, $p<0.001$). *Post hoc* analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in both fear-conditioned groups compared with their non-fear-conditioned counterparts (FC Vehicle vs NFC Vehicle [***$p<0.001$]; FC GW9662 vs NFC GW9662 [$^{++}p<0.001$]). The treatment with GW9662 did not have any significant effect on pain 2 duration in NFC or FC rats.

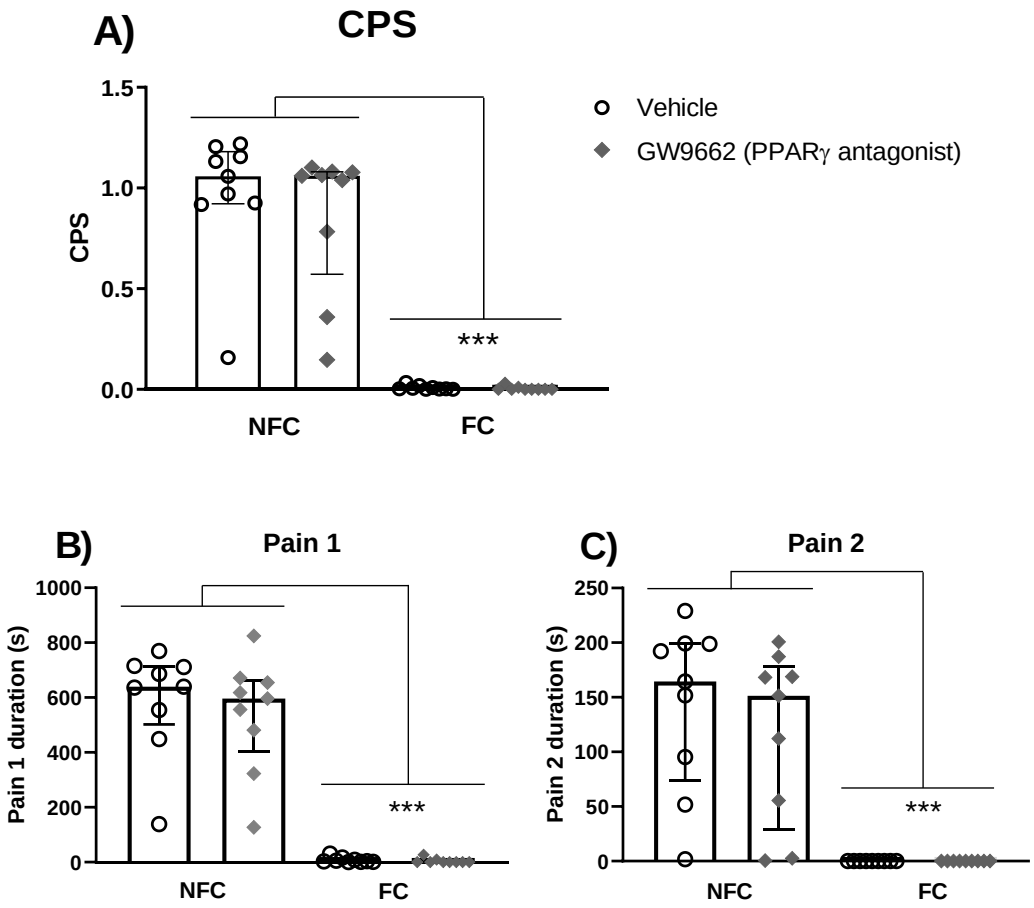


Figure 2.8: Effects of systemic administration of selective PPARγ antagonists on formalin-evoked nociceptive behaviour in non-fear conditioned (NFC) and fear conditioned (FC) rats. Post hoc analysis with Dunn's test revealed a significant difference between FC groups and their NFC counterparts [***p<0.001], in CPS values (A), Pain 1 (B) and Pain 2 (C). Data are expressed as median with interquartile range (n=9 per group).

The analysis of the CPS values in 3-min bins using Friedman's test did not show any significant effect of time (χ^2 (4) = 5.826, p>0.05) on formalin-induced nociceptive behaviour (Figure 2.19). Kruskal-Wallis test revealed a significant difference among groups in all time bins [($\chi^2_{1\text{-}3}$ (3) = 26.877, p<0.001; $\chi^2_{4\text{-}6}$ (3) = 28.186, p<0.001; $\chi^2_{7\text{-}9}$ (3) = 29.258, p<0.001; $\chi^2_{10\text{-}12}$ (3) = 28.463, p<0.001; $\chi^2_{13\text{-}15}$ (3) = 26.146, p<0.001)]. *Post hoc* analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in the FC groups compared to NFC counterparts in all time bins ($Time_{1\text{-}3}$ FC Vehicle vs NFC Vehicle, **p<0.01; $Time_{1\text{-}3}$ FC GW9662 vs NFC GW9662, $^{++}$p<0.01; $Time_{4\text{-}6}$ FC Vehicle vs NFC Vehicle, **p<0.01; $Time_{4\text{-}6}$ FC GW9662 vs NFC GW9662, $^{+++}$p<0.01; $Time_{7\text{-}9}$ FC Vehicle

vs NFC Vehicle, $^{***}p<0.01$; $Time_{7-9}$ FC GW9662 vs NFC GW9662, $^{++}p<0.01$; $Time_{10-12}$ FC Vehicle vs NFC Vehicle, $^{**}p<0.01$; $Time_{10-12}$ FC GW9662 vs NFC GW9662, $^{+++}p<0.01$; $Time_{13-15}$ FC Vehicle vs NFC Vehicle, $^{**}p<0.01$; $Time_{13-15}$ FC GW9662 vs NFC GW9662, $^{+++}p<0.01$). The treatment with GW9662 did not affect formalin-induced nociceptive behaviour in NFC or FC rats.

The analysis of paw oedema using two-way ANOVA revealed no significant effect of fear-conditioning [$F(1, 32) = 2.627$, $p>0.05$] or treatment [$F(1, 32) = 1.026$, $p>0.05$] (Figure 2.20).

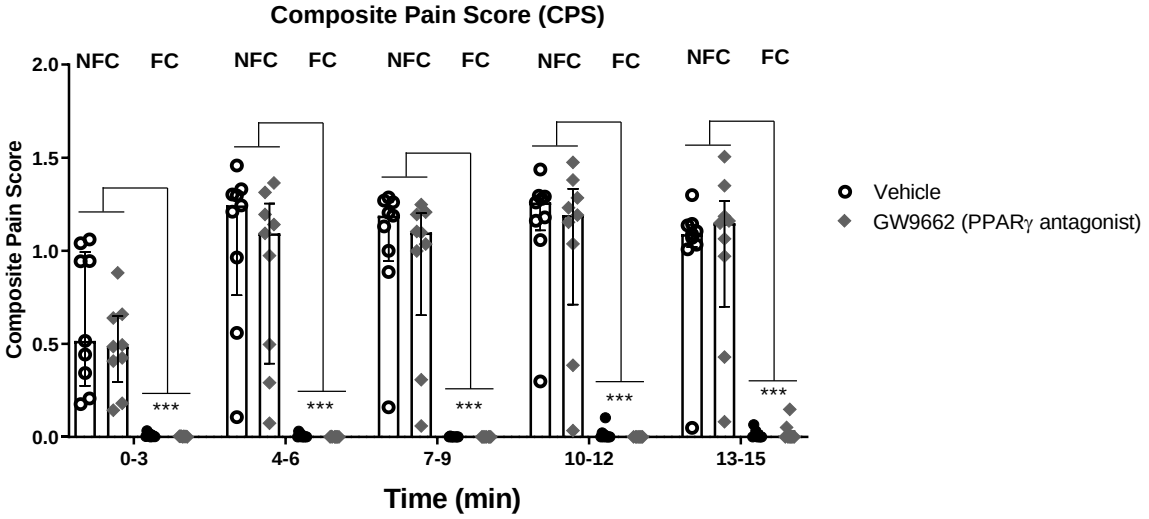


Figure 2.169: Temporal profile of the effects of systemic administration of selective PPARγ antagonist on formalin-evoked nociceptive behaviour in non-fear conditioned (NFC) and fear conditioned (FC) rats. Post hoc analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in the FC groups compared to NFC counterparts in all time bins (***$p<0.001$). Data are expressed as median with interquartile range, n=9 rats per group.

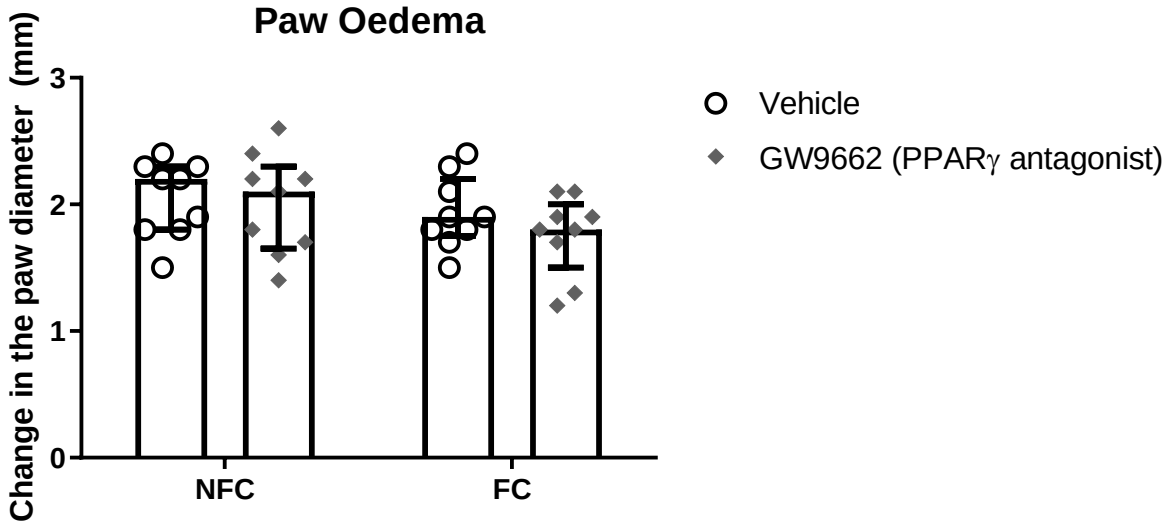


Figure 2.20: Effects of systemic administration of selective PPARγ antagonist on formalin-evoked hind paw oedema in non-fear conditioned (NFC) and fear conditioned (FC) rats. Data are expressed as mean ± S.E.M, n=9 rats per group.

2.3.2.2 Effects of systemic administration of GW9662 on fear-related behaviour in formalin-treated rats

The analysis of the freezing duration using two-way repeated-measures ANOVA revealed a significant of time [$F(2.586, 82.768) = 8.754$, $p<0.001$], conditioning [$F(1, 32) = 184.373$, $^{a}p<0.001$], time x conditioning [$F(2.586, 82.768) = 7.978$, $p<0.001$] and treatment [$F(1, 32) = 4.952$, $p>0.05$] but not of time x treatment [$F(2.586, 82.768) = 0.099$, $p>0.05$], conditioning x treatment [$F(1, 32) = 4.013$, $p>0.05$] and time x conditioning x treatment [$F(2.586, 82.768) = 0.202$, $p>0.05$] (Figure 2.21). *Post hoc* analysis with Student Newman-Keuls revealed that treatment with GW9662 in FC rats significantly affected freezing duration in two of the 3-min time bins: 1-3 and 13-15 ($^{\#}p<0.05$). *Post hoc* analysis with Student Newman-Keuls also confirmed the conditioning effects on all treatment groups and in all time bins.

The analysis of the number of faecal pellets excreted (defecation) using Kruskal-Wallis comparisons revealed a significant difference among groups ($\chi^2(3) = 28.63$, $p<0.001$) (Figure 2.22). *Post hoc* analysis with Dunn's test indicated that the number of faecal pellets excreted was significantly higher in FC groups compared to NFC counterparts [FC Vehicle

vs NFC Vehicle, **p<0.01; FC GW9662 vs NFC GW9662, ###p<0.001]. The treatment with GW9662 did not significantly alter defecation in NFC or FC rats.

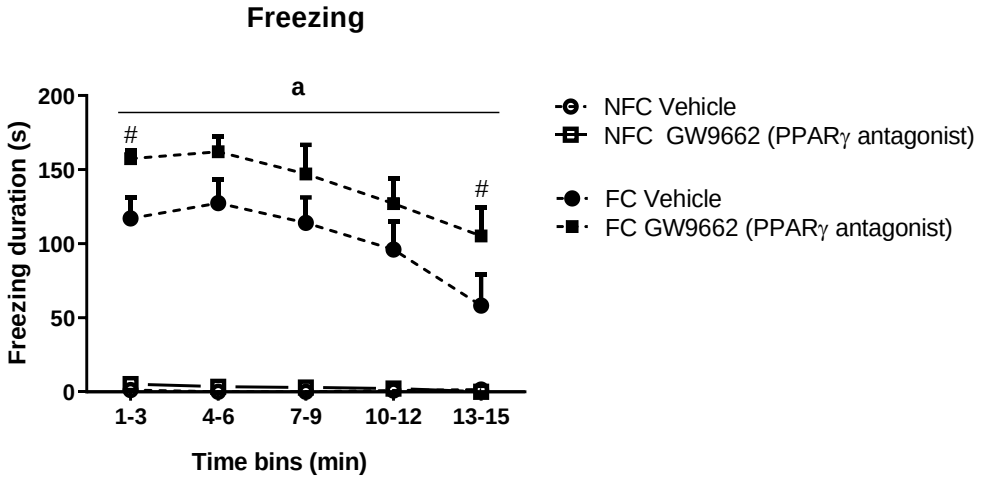


Figure 2.171: Temporal profile of the effects of systemic administration of selective PPARγ antagonist on freezing duration in NFC and FC rats. Two-way ANOVA revealed a significant effect of conditioning ([a]p<0.001) on freezing duration (Figure 2.16A). Post hoc analysis revealed that treatment with GW9662 in FC rats significantly affected freezing duration in two time bins ([#]p<0.05, vs FC Vehicle). Data are expressed as 3 minutes bins (mean ± S.E.M, n=9 rats per group).

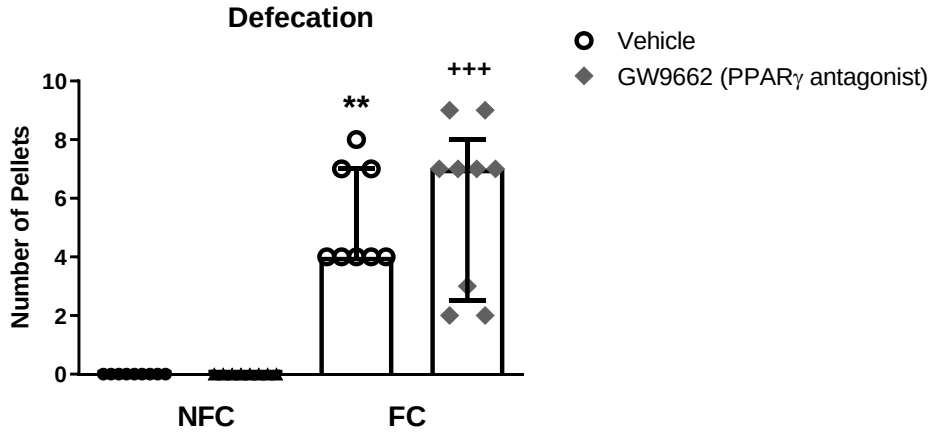


Figure 2.2: Effects of systemic administration of selective PPARγ antagonist on defecation in non-fear conditioned (NFC) and fear conditioned (FC) rats. Post hoc analysis indicated that the number of faecal pellets excreted was significantly higher in FC groups compared to NFC counterparts (**p<0.01 vs NFC Vehicle; [+++]p<0.001 vs NFC GW9662). Data are expressed as median with interquartile range, n=9 rats per group.

2.3.2.3 Effects of systemic administration of GW9662 on general/motor behaviour

The analysis of walking duration using Friedman's test revealed a significant effect of time (χ^2 (3) = 46.115, p>0.05) (Figure 2.23A). Kruskal-Wallis test revealed a significant difference among groups in all time bins [($\chi^2_{1\text{-}3}$ (3) = 27.047, p<0.001; $\chi^2_{4\text{-}6}$ (3) = 27.940, p<0.001; $\chi^2_{7\text{-}9}$ (3) = 28.741, p<0.001; $\chi^2_{10\text{-}12}$ (3) = 20.982, p<0.001; $\chi^2_{13\text{-}15}$ (3) = 14.452, p<0.01)]. *Post hoc* analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in the FC groups compared to NFC counterparts in all time bins ($Time_{1\text{-}3}$ FC Vehicle vs NFC Vehicle, $^{**}p<0.01$; $Time_{1\text{-}3}$ FC GW9662 vs NFC GW9662, $^{+++}p<0.01$; $Time_{4\text{-}6}$ FC Vehicle vs NFC Vehicle, $^{**}p<0.01$; $Time_{4\text{-}6}$ FC GW9662 vs NFC GW9662, $^{+++}p<0.01$; $Time_{7\text{-}9}$ FC Vehicle vs NFC Vehicle, $^{**}p<0.01$; $Time_{7\text{-}9}$ FC GW9662 vs NFC GW9662, $^{+++}p<0.01$; $Time_{10\text{-}12}$ FC Vehicle vs NFC Vehicle, $^{*}p<0.01$; $Time_{10\text{-}12}$ FC GW9662 vs NFC GW9662, $^{++}p<0.01$; $Time_{13\text{-}15}$ FC Vehicle vs NFC Vehicle, $^{*}p<0.01$). The treatment with GW9662 did not affect walking duration in NFC or FC rats.

The analysis of grooming duration using Friedman's test revealed a significant effect of time (χ^2 (3) = 7.597, p>0.05) (Figure 2.23B). Kruskal-Wallis test revealed a significant difference among groups in one time bin [($\chi^2_{1\text{-}3}$ (3) = 16.478, p<0.01]. *Post hoc* analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in the FC groups compared to NFC counterparts in all time bins ($Time_{1\text{-}3}$ FC Vehicle vs NFC Vehicle, $^{**}p<0.01$). The treatment with GW9662 did not affect grooming duration in NFC or FC rats.

The analysis of rearing duration using Friedman's test revealed a significant effect of time (χ^2 (3) = 6.784, p>0.05) (Figure 2.23C). Kruskal-Wallis did not show any significant difference among groups in any of the time bins [$\chi^2_{1\text{-}3}$ (3) = 4.669, p>0.05; $\chi^2_{4\text{-}6}$ (3) = 0.267, p>0.05; $\chi^2_{7\text{-}9}$ (3) = 1.175, p>0.05; $\chi^2_{10\text{-}12}$ (3) = 0.267, p>0.05; $\chi^2_{13\text{-}15}$ (3) = 3.198, p>0.05].

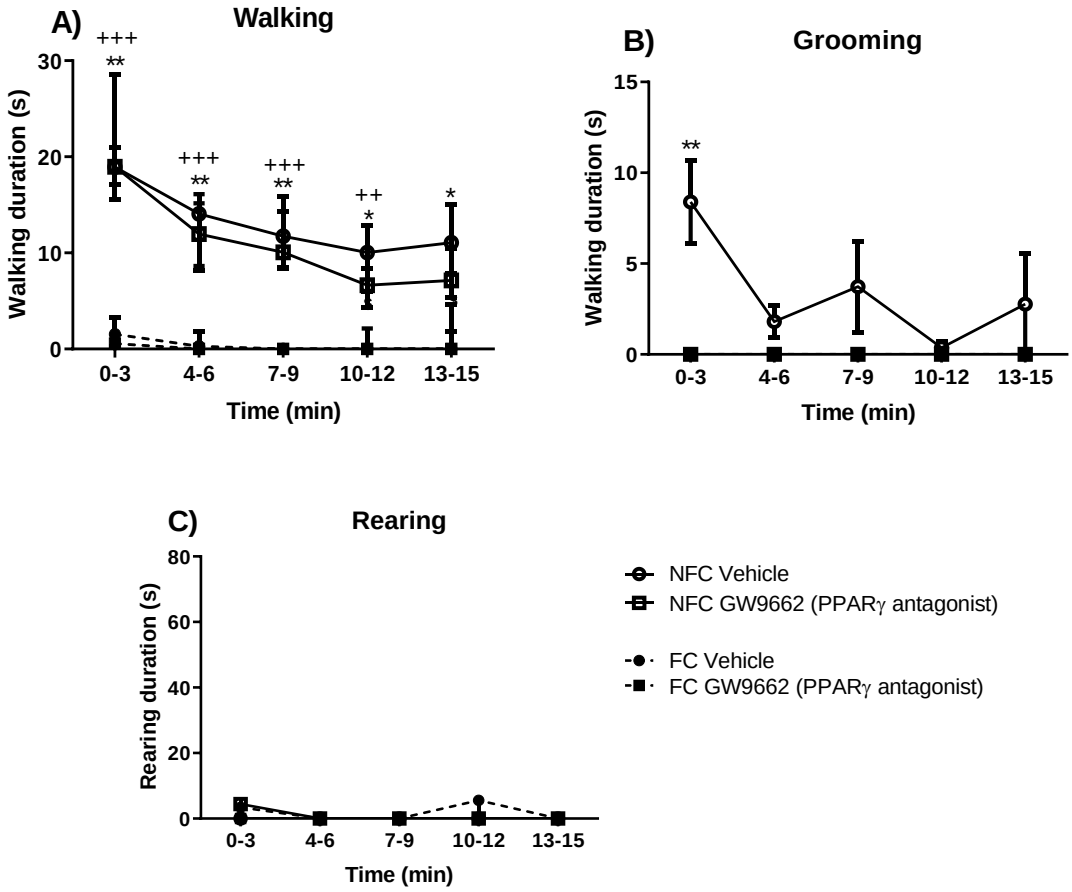


Figure 2.183: Temporal profile of the effects of fear-conditioning and systemic administration of selective PPARγ antagonist on walking duration (A), grooming duration (B), and rearing duration (C). Post hoc analysis with Dunn's test indicated that formalin-evoked nociceptive behaviour was significantly lower in the FC groups compared to NFC counterparts in all time bins (**$p<0.01$ and *$p<0.05$, vs NFC Vehicle; $^{++}p<0.01$ and $^{+++}p<0.001$, vs NFC GW9662). Data are expressed as median with interquartile range and min/max, n=9 rats per group.

2.3.2.4 Effect of fear conditioning and GW9662 administration on neurotransmitter levels in the BLA, CeA, and VH of formalin treated rats

The PPAR signalling system has previously been shown to be associated with positive changes in the GABAergic (Sasso et al., 2010), promotes an increase in the expression of glutamatergic receptors (Ching et al., 2015), and increased levels of serotonin (Waku et al., 2010; Mijangos-Moreno et al., 2016) and dopamine (Mascia et al., 2011; Mijangos-Moreno et al., 2016b; Chikahisa et al., 2019). Therefore, the blockade of PPARs could affect levels of one or more of these neurotransmitters, which it turn play key roles in pain and fear. In order to check if the alterations in pain and/or fear responses after fear conditioning and treatment with PPAR antagonists were associated with changes in the levels of neurotransmitters, we examined tissue levels of GABA, glutamate, serotonin and dopamine in the BLA, CeA, and VH.

2.3.2.4.1 Effect of fear conditioning and GW9662 administration on neurotransmitter levels in the BLA

The analysis of the levels of GABA in the BLA using three-way ANOVA revealed that side [$F(1, 56) < 0.001$, $p>0.05$], fear conditioning [$F(1, 56) = 0.046$, $p>0.05$] and treatment [$F(1, 56) = 1.130$, $p>0.05$] did not have any effect on GABA levels (Figure 2.24A). There were no significant effects of treatment x conditioning [$F(1, 56) = 0.306$, $p>0.05$], treatment x side [$F(1, 56) < 0.001$, $p>0.05$], conditioning x side [$F(1, 56) = 0.293$, $p>0.05$] and treatment x conditioning x side [$F(1, 56) = 3.255$, $p>0.05$] on GABA levels in the BLA. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right $F(1, 25) = 0.397$, $p>0.05$; Left $F(1, 31) = 0.364$, $p>0.05$], fear conditioning [Right $F(1, 25) = 0.448$, $p>0.05$; Left $F(1, 31) = 0.240$, $p>0.05$], or treatment x fear conditioning [Right $F(1, 25) = 1.799$, $p>0.05$; Left $F(1, 31) = 0.749$, $p>0.05$] on GABA levels.

The analysis of the levels of glutamate in the BLA using three-way ANOVA revealed an overall effect of side [$F(1, 51) = 7.135$, $p=0.010$] (Figure 2.24B). However, *post hoc* pairwise group comparisons did not reach statistical significance. There were no effects of fear conditioning [$F(1, 51) = 0.289$, $p>0.05$] and treatment [$F(1, 51) = 0.292$, $p>0.05$], conditioning x side [$F(1, 51) = 0.611$, $p>0.05$], treatment x conditioning [$F(1, 51) = 1.275$, $p>0.05$], treatment x side [$F(1, 51) = 1.061$] and treatment x conditioning x side [$F(1, 51)$

= 2.218, p>0.05] on glutamate levels in the BLA. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (1, 21) = 0.127, p>0.05; Left F (1, 30) = 1.301, p>0.05], fear conditioning [Right F (1, 21) = 0.920, p>0.05; Left F (1, 30) = 0.032, p>0.05], or treatment x fear conditioning [Right F (1, 21) = 3.626, p>0.05; Left F (1, 30) = 0.068, p>0.05] on glutamate levels.

The analysis of the levels of serotonin in the BLA using three-way ANOVA revealed that side [F (1, 61) < 0.752, p>0.05], fear conditioning [F (1, 61) = 1.445, p>0.05] and treatment [F (1, 61) = 0.327, p>0.05] did not have any effect on the levels of serotonin in the BLA (Figure 2.24C). There were no significant effects of treatment x conditioning [F (1, 61) = 2.087, p>0.05], treatment x side [F (1, 61) = 0.093, p>0.05], conditioning x side [F (1, 61) = 0.082, p>0.05] and treatment x conditioning x side [F (1, 61) = 2.282, p>0.05] on serotonin levels in the BLA. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (1, 31) = 0.052, p>0.05; Left F (1, 30) = 0.002, p>0.05], fear conditioning [Right F (1, 31) = 0.602, p>0.05; Left F (1, 30) = 1.312, p>0.05], or treatment x fear conditioning [Right F (1, 31) = 3.069, p>0.05; Left F (1, 30) = 0.151, p>0.05] on serotonin levels.

The analysis of the levels of dopamine in the BLA using Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (7) = 24.558, p<0.001) (Figure 2.24D). *Post hoc* analysis with Dunn's test indicated that the dopamine levels were significantly higher in the right NFC GW9662 compared to left counterparts [Right NFC GW9662 vs Left NFC GW9662, $^{+}$p<0.05]. When each side was analysed separately, Kruskal Wallis did not show any significant difference among group in the right [χ^2 (3) = 2.301, p>0.05] or in the left [χ^2 (3) = 0.566, p>0.05] sides in dopamine levels in the BLA.

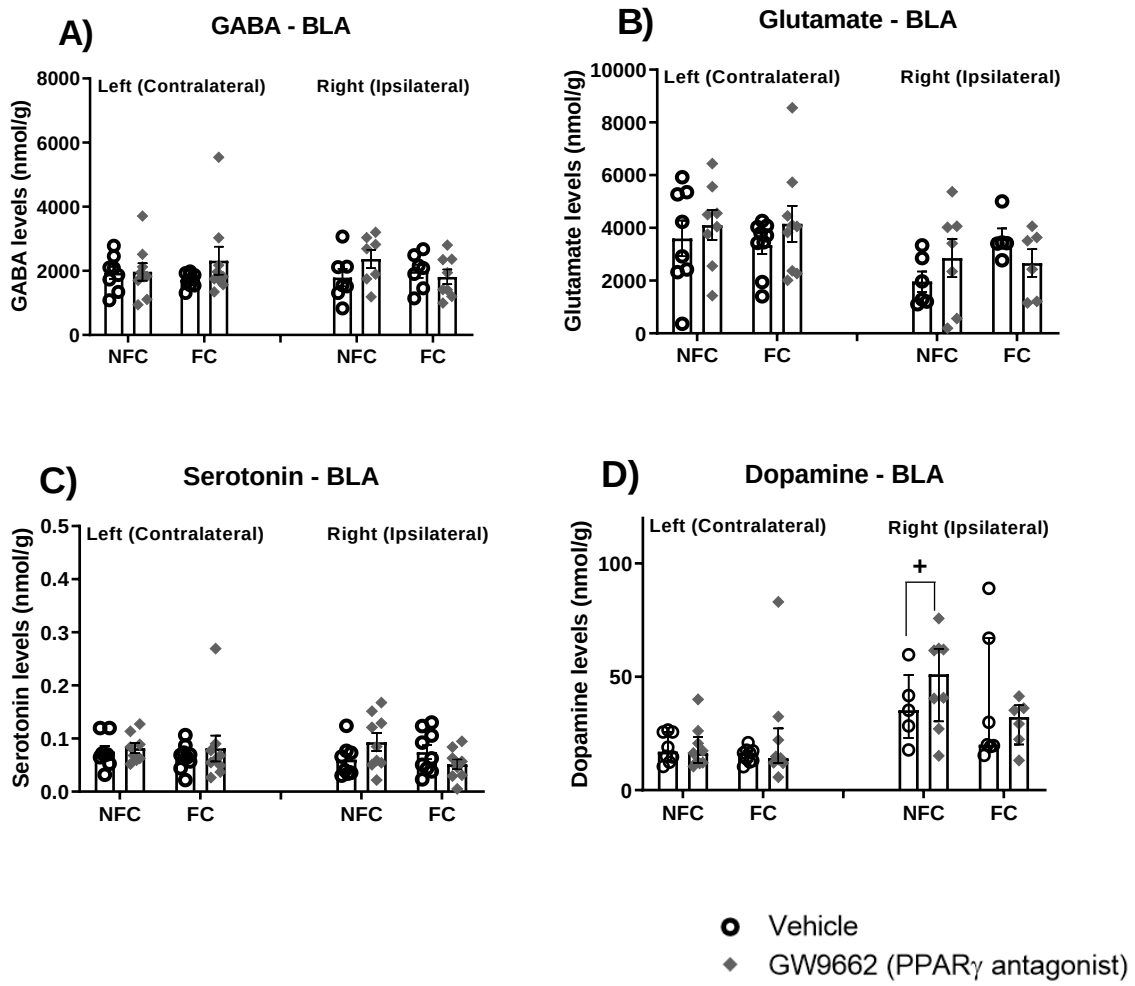


Figure 2.4: Effects of systemic administration of a selective PPARγ antagonist on GABA (A), glutamate (B), serotonin (C), and dopamine (D) levels in the basolateral amygdala (BLA) in NFC and FC rats. Post hoc analysis indicated that the dopamine levels were significantly higher in the Right NFC GW9662 ($^{+}p<0.05$, vs Left NFC GW9662). Data are expressed as mean ± S.E.M (A, B and C) and as median with interquartile range (D), n=7-9 rats per group.

2.3.2.4.2 Effect of fear conditioning and GW9662 administration on neurotransmitter levels in the CeA

The analysis of the levels of GABA in the CeA using three-way ANOVA revealed an overall effect of side [F (1, 61) = 10.744, $^{a}p = 0.002$] and fear conditioning [F (1, 61) = 5.634, p=0.021] (Figure 2.25A). However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. There were no significant effects of treatment [F (1, 61) = 1.216, p>0.05] conditioning x side [F (1, 61) = 2.419, p>0.05], treatment x conditioning [F (1, 61) = 3.222, p>0.05], treatment x side [F (1, 61) = 0.178] and treatment x conditioning x side [F (1, 61) = 0.223, p>0.05] on GABA levels in the CeA. When each side was analysed separately, two-way ANOVA revealed an effect of fear conditioning on GABA levels in the right [Right F (1, 31) = 6.141, p<0.05] but not in the left side [Left F (1, 30) = 0.462, p>0.05]. Treatment [Right F (1, 31) = 0.185, p>0.05; Left F (1, 30) = 1.600, p>0.05], or treatment x fear conditioning [Right F (1, 31) = 2.046, p>0.05; Left F (1, 30) = 1.205, p>0.05] did not have any effect on GABA levels in the CeA. *Post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance.

The analysis of the levels of glutamate in the CeA using three-way ANOVA revealed an overall effect of side [F (1, 61) = 16.776, $^{a}p<0.001$] (Figure 2.25B). However, *post hoc* pairwise group comparisons did not reach statistical significance. There were no effects of fear conditioning [F (1, 61) = 0.009, p>0.05], treatment [F (1, 61) = 0.198, p>0.05], conditioning x side [F (1, 61) = 0.012, p>0.05], treatment x conditioning [F (1, 61) = 0.100, p>0.05], treatment x side [F (1, 61) < 0.001] and treatment x conditioning x side [F (1, 61) = 0.179, p>0.05] on glutamate levels in the CeA. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (1, 31) = 0.091, p>0.05; Left F (1, 30) = 0.107, p>0.05], fear conditioning [Right F (1, 31) < 0.001, p>0.05; Left F (1, 30) = 0.020, p>0.05], or treatment x fear conditioning [Right F (1, 31) = 0.006, p>0.05; Left F (1, 30) = 0.268, p>0.05] on glutamate levels.

The analysis of the levels of serotonin in the CeA using three-way ANOVA revealed an overall effect of side [F (1, 59) = 35.470, $^{a}p<0.001$] (Figure 2.25C). *Post hoc* pairwise group comparisons with Student Newman-Keuls test showed that levels of serotonin are significantly higher in the right side compared to the left side of NFC vehicle-treated rats (Right NFC Vehicle vs Left NFC Vehicle, *p<0.05) and NFC GW9662-treated (Right NFC GW9662 vs Left NFC GW9662, #p<0.05) rats. There were no significant effects of fear

conditioning [F (1, 59) = 2.215, p>0.05], treatment [F (1, 59) = 0.001, p>0.05], conditioning x side [F (1, 59) = 3.265, p>0.05], treatment x conditioning [F (1, 59) = 1.025, p>0.05], treatment x side [F (1, 61) < 0.305] and treatment x conditioning x side [F (1, 59) = 0.572, p>0.05] on serotonin levels in the CeA. When each side was analysed separately, two-way ANOVA revealed an effect of fear conditioning on serotonin levels in the right [Right F (1, 31) = 4.280, p<0.05] but not in the left side [Left F (1, 30) = 0.077, p>0.05]. *Post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. There were no effects of treatment [Right F (1, 31) = 0.134, p>0.05; Left F (1, 30) = 0.206, p>0.05], or treatment x fear conditioning [Right F (1, 31) = 1.234, p>0.05; Left F (1, 30) = 0.050, p>0.05] on serotonin levels in the CeA.

The analysis of the levels of dopamine in the CeA using three-way ANOVA revealed that there were no significant effects of side [F (1, 60) = 0.197, p>0.05], fear conditioning [F (1, 60) = 0.340, p>0.05], treatment [F (1, 60) = 3.293, p>0.05], conditioning x side [F (1, 60) = 0.773, p>0.05], treatment x conditioning [F (1, 60) = 0.471, p>0.05], treatment x side [F (1, 60) = 2.567] and treatment x conditioning x side [F (1, 60) = 1.619, p>0.05] (Figure 2.25D). When each side was analysed separately, two-way ANOVA revealed an effect of treatment on dopamine levels in the right [Right F (1, 30) = 5.317, p<0.05] but in the left side [Left F (1, 30) = 0.024, p>0.05]. *Post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. There were no effects of fear conditioning [Right F (1, 30) = 0.524, p>0.05; Left F (1, 30) = 0.177, p>0.05], or treatment x fear conditioning [Right F (1, 30) = 0.194, p>0.05; Left F (1, 30) = 2.179, p>0.05] on dopamine levels in the CeA.

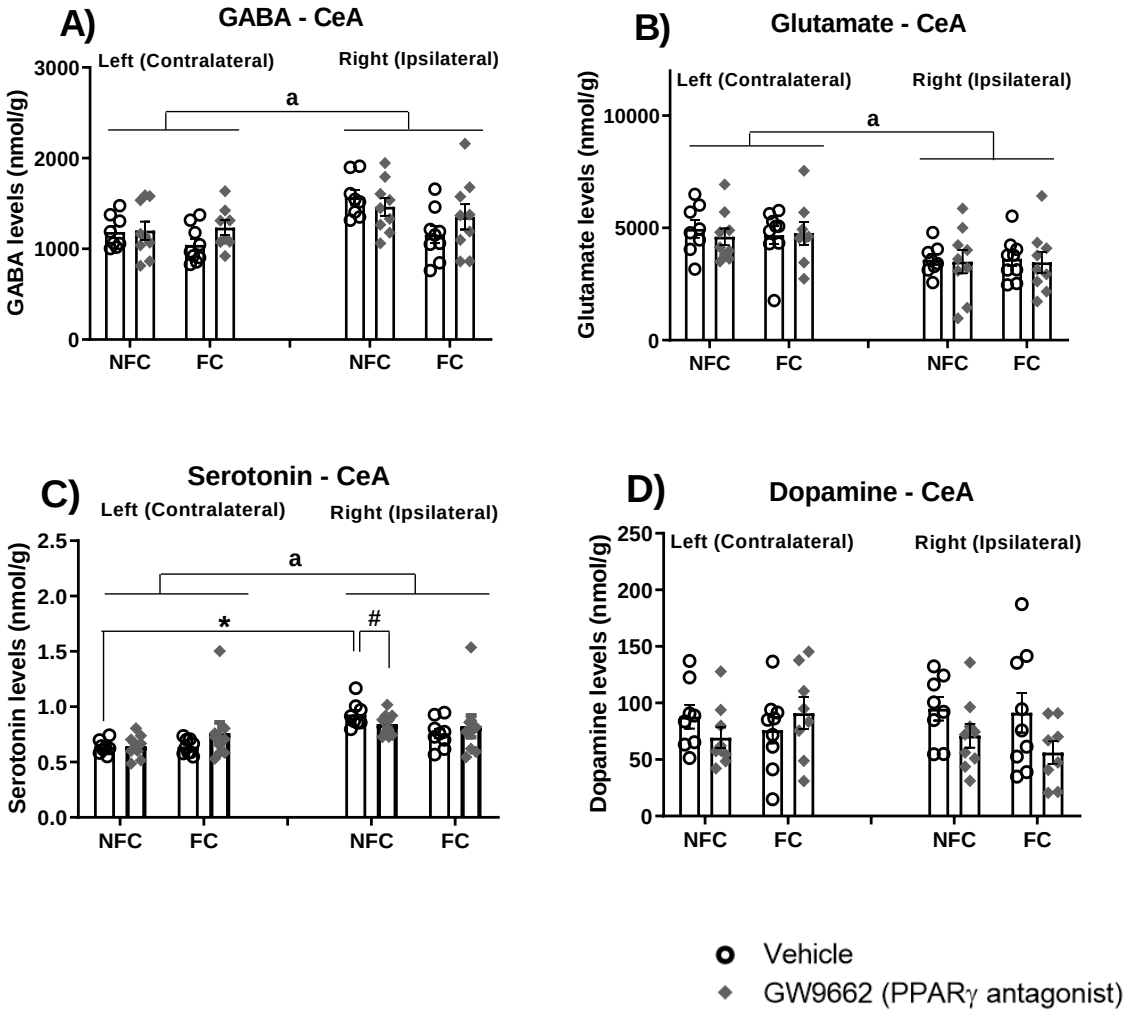


Figure 2.195: Effects of systemic administration of a selective PPARγ antagonist on GABA (A), glutamate (B), serotonin (C), and dopamine (D) levels in the central nuclei of the amygdala (CeA) on NFC and FC rats. Three-way ANOVA have shown a significant effect of side ($^{a}p<0.05$) on GABA, glutamate, and serotonin levels. Data are expressed as mean ± S.E.M, n=7-9 rats per group.

2.3.2.4.3 Effect of fear conditioning and GW9662 administration on neurotransmitter levels in the VH

The analysis of the levels of GABA in the VH using Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (7) = 17.120, $p<0.05$) (Figure 2.26A). However, *post hoc* analysis with Dunn's test did not show any significant changes in GABA levels. When each side was analysed separately, Kruskal Wallis did not show any significant

difference among group in the right [χ^2 (3) = 2.302, p>0.05] or in the left [χ^2 (3) = 2.530, p>0.05] sides in GABA levels in the VH.

The analysis of the levels of glutamate in the VH using three-way ANOVA revealed that there were significant effects of side [F (1, 54) = 0.768, p>0.05], fear conditioning [F (1, 54) = 0.052, p>0.05], treatment [F (1, 54) = 0.010, p>0.05], treatment x conditioning [F (1, 54) = 0.390, p>0.05], treatment x side [F (1, 54) = 0.063], conditioning x side [F (1, 54) = 0.197, p>0.05] and treatment x conditioning x side [F (1, 54) = 0.345, p>0.05] (Figure 2.26B). When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (1, 28) = 0.077, p>0.05; Left F (1, 26) = 0.870, p>0.05], fear conditioning [Right F (1, 28) = 0.001, p>0.05; Left F (1, 26) = 0.314, p>0.05], or treatment x fear conditioning [Right F (1, 28) = 0.438, p>0.05; Left F (1, 26) = 0.038, p>0.05] on glutamate levels.

The analysis of the levels of serotonin in the VH using Three-way ANOVA revealed an overall effect of side [F (1, 53) = 20.514, ^{a}p<0.001] (Figure 2.26C). *Post hoc* pairwise group comparisons with Student Newman-Keuls test showed that levels of serotonin are significantly higher in the left side of NFC vehicle-treated rats (Right NFC Vehicle vs Left NFC Vehicle, *p<0.05). There were no significant effects of fear conditioning [F (1, 53) = 0.598, p>0.05], treatment [F (1, 53) = 0.056, p>0.05], conditioning x side [F (1, 53) = 0.655, p>0.05], treatment x conditioning [F (1, 53) = 0.122, p>0.05], treatment x side [F (1, 53) = 0.749] and treatment x conditioning x side [F (1, 53) = 0.001, p>0.05] on serotonin levels in the VH (Figure 2.24C). When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (1, 28) = 0.289, p>0.05; Left F (1, 25) = 0.373, p>0.05], fear conditioning [Right F (1, 28) = 0.088, p>0.05; Left F (1, 25) = 1.799, p>0.05], or treatment x fear conditioning [Right F (1, 28) = 0.355, p>0.05; Left F (1, 26) < 0.001, p>0.05] on serotonin levels.

The analysis of the levels of dopamine in the VH using three-way ANOVA revealed an overall effect of side [F (1, 53) = 12.908, ^{a}p<0.001] (Figure 2.26D). However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. There were no effects of fear conditioning [F (1, 53) = 2.917, p>0.05], treatment [F (1, 53) = 0.008, p>0.05], conditioning x side [F (1, 53) = 0.004, p>0.05], treatment x conditioning [F (1, 53) = 0.812, p>0.05], treatment x side [F (1, 53) = 3.629] and treatment x conditioning x side [F (1, 53) = 0.154, p>0.05] on dopamine levels in the

VH. When each side was analysed separately, two-way ANOVA did not reveal any effect of treatment [Right F (1, 29) = 1.019, p>0.05; Left F (1, 24) = 3.515, p>0.05], fear conditioning [Right F (1, 29) = 0.546, p>0.05; Left F (1, 24) = 1.119, p>0.05], or treatment x fear conditioning [Right F (1, 29) = 0.595, p>0.05; Left F (1, 24) = 0.924, p>0.05] on dopamine levels.

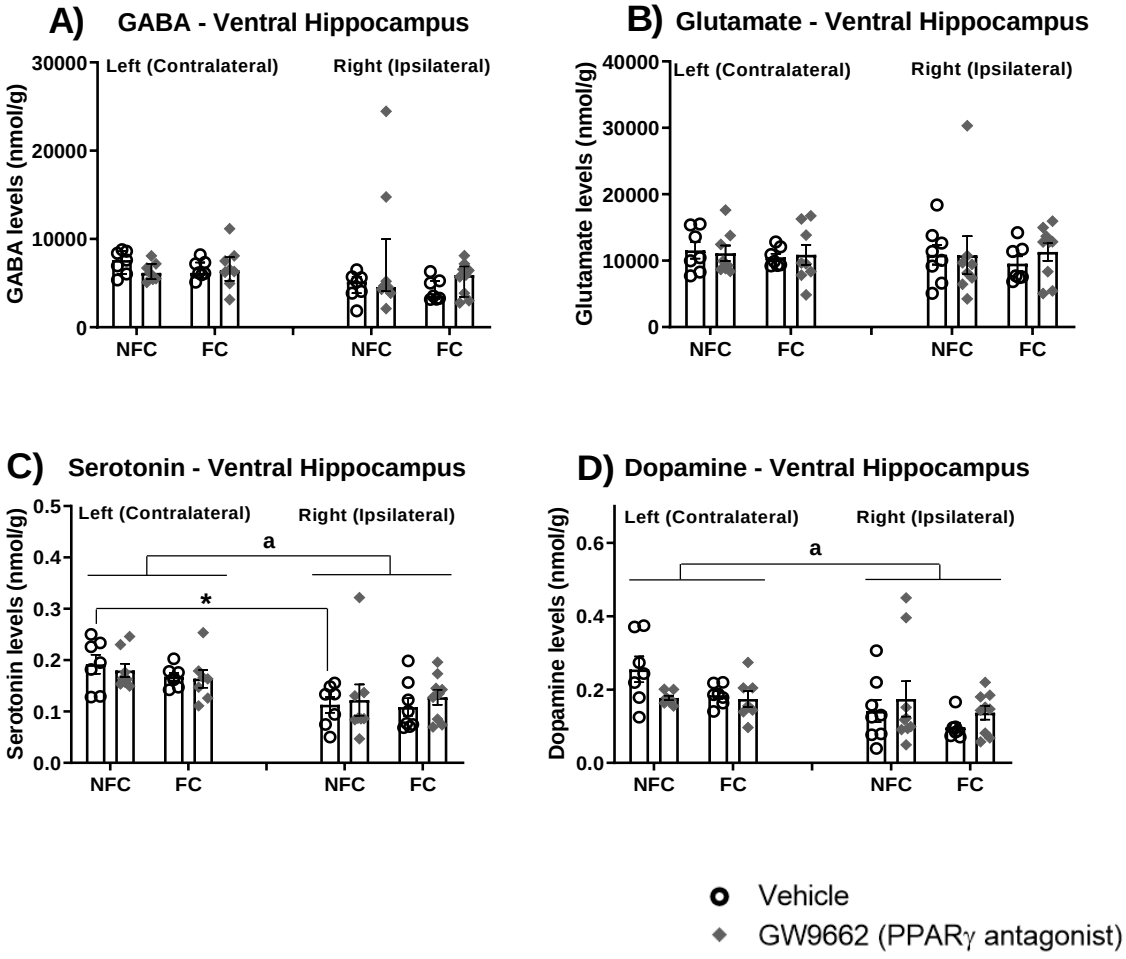


Figure 2.206: Effects of systemic administration of a selective PPARγ antagonist on GABA (A), glutamate (B), serotonin (C), and dopamine (D) levels in the ventral hippocampus (VH) of NFC and FC rats. Three-way ANOVA revealed a significant effect of side ($^{a}p<0.05$) on serotonin and dopamine levels. Post hoc showed that levels of serotonin are significantly higher in the left side of NFC vehicle-treated rats (*p<0.05). Data are expressed as mean ± S.E.M (B, C, and D) and as median with interquartile range (A), n=7-9 rats per group.

2.3.2.5 Effect of fear conditioning and GW9662 administration on endocannabinoid and NAE levels in the BLA, CeA, and VH of formalin treated rats

In order to check if the alterations in pain and/or fear responses after fear conditioning and treatment with PPAR antagonists were associated with changes in the levels of the endogenous ligands (i.e. NAEs) and endocannabinoids, we examined tissue levels of 2-AG, AEA, PEA and OEA in the BLA, CeA, and VH.

2.3.2.5.1 Effect of fear conditioning and GW9662 administration on endocannabinoid and NAE levels in the BLA

The analysis of the levels of 2-AG in the BLA using three-way ANOVA revealed that there were no effects of side [$F(1, 53) = 1.350$, $p>0.05$], fear conditioning [$F(1, 53) = 1.627$, $p>0.05$], treatment [$F(1, 53) = 0.224$, $p>0.05$], conditioning x side [$F(1, 53) = 0.285$, $p>0.05$], treatment x conditioning [$F(1, 53) = 1.289$, $p>0.05$], treatment x side [$F(1, 53) = 0.243$] and treatment x conditioning x side [$F(1, 53) = 3.146$, $p>0.05$] (Figure 2.27A). When each side was analysed separately, two-way ANOVA revealed an effect of the interaction of treatment x fear conditioning on 2-AG levels in the left [Left $F(1, 27) = 5.393$, $p<0.05$] but not in the right [Right $F(1, 26) = 0.166$, $p>0.05$]. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. There were no effects of fear conditioning [Right $F(1, 26) = 0.224$, $p>0.05$; Left $F(1, 27) = 2.086$, $p>0.05$], or treatment [Right $F(1, 26) < 0.001$, $p>0.05$; Left $F(1, 27) = 0.595$, $p>0.05$] on 2-AG levels.

The analysis of the levels of AEA in the BLA using three-way ANOVA revealed that there were no significant effects of side [$F(1, 53) = 0.167$, $p>0.05$], fear conditioning [$F(1, 53) = 3.235$, $p>0.05$], treatment [$F(1, 53) = 0.360$, $p>0.05$], conditioning x side [$F(1, 53) < 0.001$, $p>0.05$], treatment x conditioning [$F(1, 53) = 0.005$, $p>0.05$], treatment x side [$F(1, 53) = 1.713$] (Figure 2.27B). Treatment x conditioning x side [$F(1, 53) = 3.146$, $p=0.005$] interaction significantly affected AEA levels in the BLA. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. When each side was analysed separately, two-way ANOVA revealed an effect of the interaction of treatment x fear conditioning in the right [Right $F(1, 24) = 5.856$, $p<0.05$] but not in the left [Left $F(1, 29) = 3.504$, $p>0.05$] on AEA levels. However, *post*

hoc pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. There were no significant effects of fear conditioning [Right $F(1, 24) = 2.143$, $p>0.05$; Left $F(1, 29) = 1.411$, $p>0.05$], or treatment [Right $F(1, 24) = 0.332$, $p>0.05$; Left $F(1, 29) = 1.593$, $p>0.05$] on AEA levels.

The analysis of the levels of PEA in the BLA using three-way ANOVA revealed that there were no significant effects of side [$F(1, 51) = 1.873$, $p>0.05$], fear conditioning [$F(1, 51) = 0.036$, $p>0.05$], treatment [$F(1, 51) = 1.987$, $p>0.05$], treatment x conditioning [$F(1, 51) = 0.228$, $p>0.05$], treatment x side [$F(1, 51) = 0.021$] and treatment x conditioning x side [$F(1, 51) = 2.112$, $p>0.05$] (Figure 2.27C). The interaction of conditioning x side [$F(1, 51) = 5.508$, $p=0.023$] significantly affected PEA levels in the BLA. However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. When each side was analysed separately, two-way ANOVA did not reveal any effect of the treatment [Right $F(1, 28) = 1.037$, $p>0.05$; Left $F(1, 23) = 0.936$, $p>0.05$], fear conditioning [Right $F(1, 28) = 3.013$, $p>0.05$; Left $F(1, 23) = 2.493$, $p>0.05$], or treatment x conditioning [Right $F(1, 28) = 0.616$, $p>0.05$; Left $F(1, 23) = 1.446$, $p>0.05$] on PEA levels in the BLA.

The analysis of the levels of OEA in the BLA using three-way ANOVA revealed an overall effect of side [$F(1, 61) = 5.047$, $^{a}p = 0.028$] (Figure 2.27D). However, *post hoc* pairwise group comparisons with Student Newman-Keuls test did not reach statistical significance. There were no significant effects of fear conditioning [$F(1, 61) = 0.686$, $p>0.05$], treatment [$F(1, 61) = 1.231$, $p>0.05$], conditioning x side [$F(1, 61) = 0.069$, $p>0.05$], treatment x conditioning [$F(1, 61) = 1.764$, $p>0.05$], treatment x side [$F(1, 61) = 0.363$] and treatment x conditioning x side [$F(1, 61) = 0.040$, $p>0.05$] on OEA levels in the BLA. When each side was analysed separately, Kruskal Wallis did not show any significant difference among group in the right [$\chi^2(3) = 1.687$, $p>0.05$] or in the left [$\chi^2(3) = 2.830$, $p>0.05$] sides in OEA levels in the BLA.

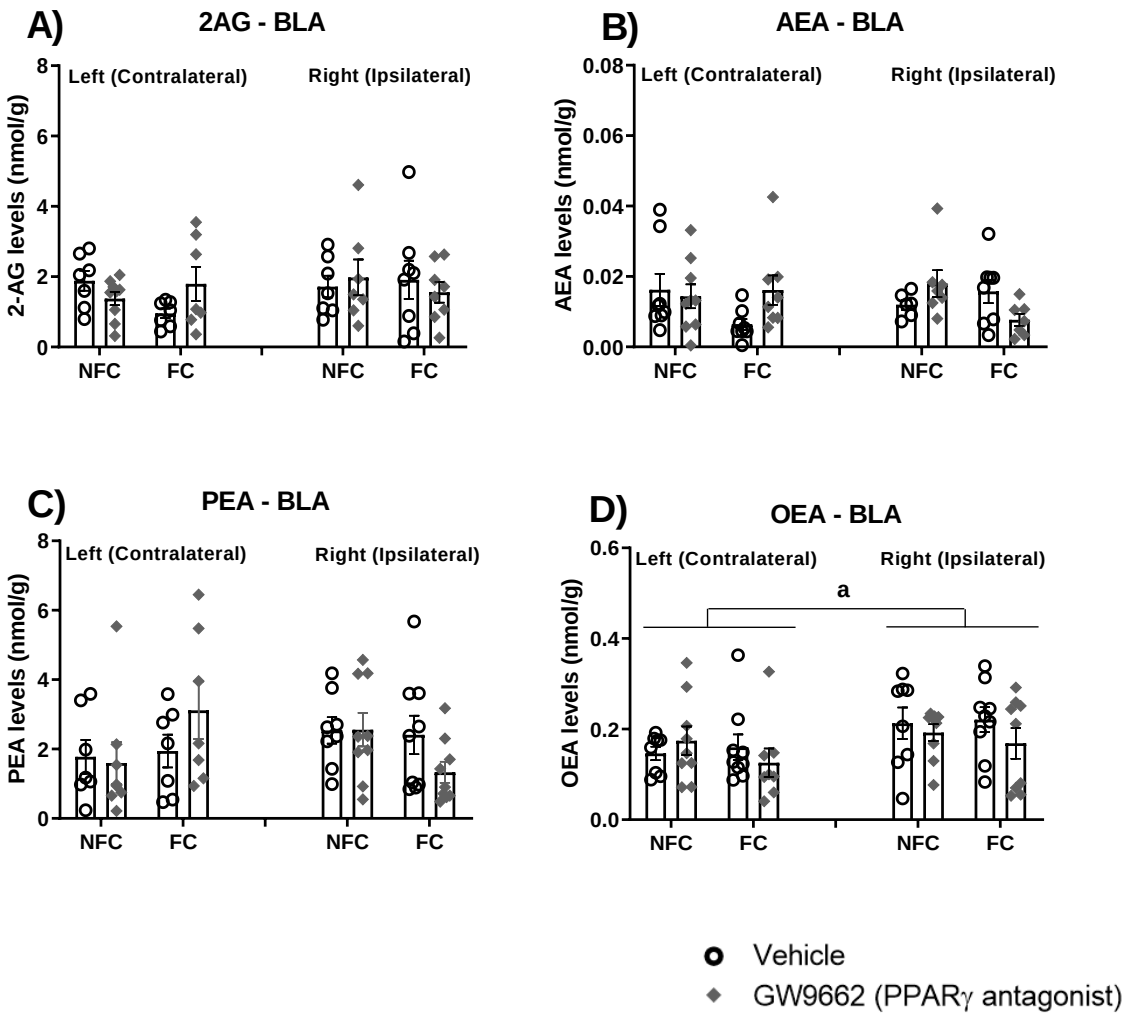


Figure 2.217: Effects of systemic administration of a selective PPARγ antagonist on 2-AG (A), AEA (B), PEA (C), and OEA (D) levels in the basolateral amygdala (BLA) in NFC and FC rats. Three-way ANOVA revealed a significant effect of side ($^{a}p<0.05$) on OEA levels. Data are expressed as mean ± S.E.M, n=7-9 rats per group.

2.3.2.5.2 Effect of fear conditioning and GW9662 administration on endocannabinoid and NAE levels in the CeA

The analysis of the levels of 2-AG in the CeA using Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (7) = 38.506, $p<0.001$) (Figure 2.28A). *Post hoc* analysis with Dunn's test revealed that 2-AG levels are significantly higher in the right side in both NFC groups compared to their left counterparts [Right NFC Vehicle vs Left NFC Vehicle, $^{**}p<0.01$; Right NFC GW9662 vs Left NFC GW9662, $^{+}p<0.05$]. When each side was analysed separately, Kruskal Wallis did not show any significant difference

among group in the right [χ^2 (3) = 2.998, p>0.05] or in the left [χ^2 (3) = 2.103, p>0.05] 2-AG levels in the CeA.

The analysis of the levels of AEA in the CeA using Kruskal-Wallis comparisons did not reveal any significant differences among groups (χ^2 (7) = 6.673, p<0.001 (Figure 2.28B). When each side was analysed separately, Kruskal Wallis did not show any significant difference among group in the right [χ^2 (3) = 1.952, p>0.05] or in the left [χ^2 (3) = 3.756, p>0.05] AEA levels in the CeA.

The analysis of the levels of PEA in the CeA using Three-way ANOVA revealed that there were no effects of side [F (1, 30) = 0.640, p>0.05], fear conditioning [F (1, 30) = 0.158, p>0.05], treatment [F (1, 30) = 0.012, p>0.05], conditioning x side [F (1, 30) = 0.033, p>0.05], treatment x conditioning [F (1, 30) = 0.092, p>0.05], treatment x side [F (1, 30) = 1.019] and treatment x conditioning x side [F (1, 30) = 0.535, p>0.05] (Figure 2.28C). When each side was analysed separately, two-way ANOVA did not reveal any effect of the treatment [Right F (1, 20) = 0.812, p>0.05; Left F (1, 20) = 0.361, p>0.05], fear conditioning [Right F (1, 20) = 0.218, p>0.05; Left F (1, 20) = 0.021, p>0.05], or treatment x conditioning [Right F (1, 20) = 0.695, p>0.05; Left F (1, 20) = 0.082, p>0.05] on PEA levels in the CeA.

The analysis of the levels of OEA in the CeA using three-way ANOVA revealed an overall effect of side [F (1, 53) = 4.699, ^{a}p=0.035] (Figure 2.28D). However, *post hoc* pairwise group comparisons did not reach statistical significance. There were no significant effects of fear conditioning [F (1, 53) = 0.052, p>0.05], treatment [F (1, 53) = 0.324, p>0.05], conditioning x side [F (1, 53) = 0.536, p>0.05], treatment x conditioning [F (1, 53) = 0.052, p>0.05], treatment x side [F (1, 53) = 0.189] and treatment x conditioning x side [F (1, 53) = 0.041, p>0.05] on OEA levels in the CeA. When each side was analysed separately, two-way ANOVA did not reveal any effect of the treatment [Right F (1, 28) = 0.009, p>0.05; Left F (1, 25) = 0.490, p>0.05], fear conditioning [Right F (1, 28) = 0.476, p>0.05; Left F (1, 25) = 0.124, p>0.05], or treatment x conditioning [Right F (1, 28) < 0.001, p>0.05; Left F (1, 25) = 0.090, p>0.05] on OEA levels in the CeA.

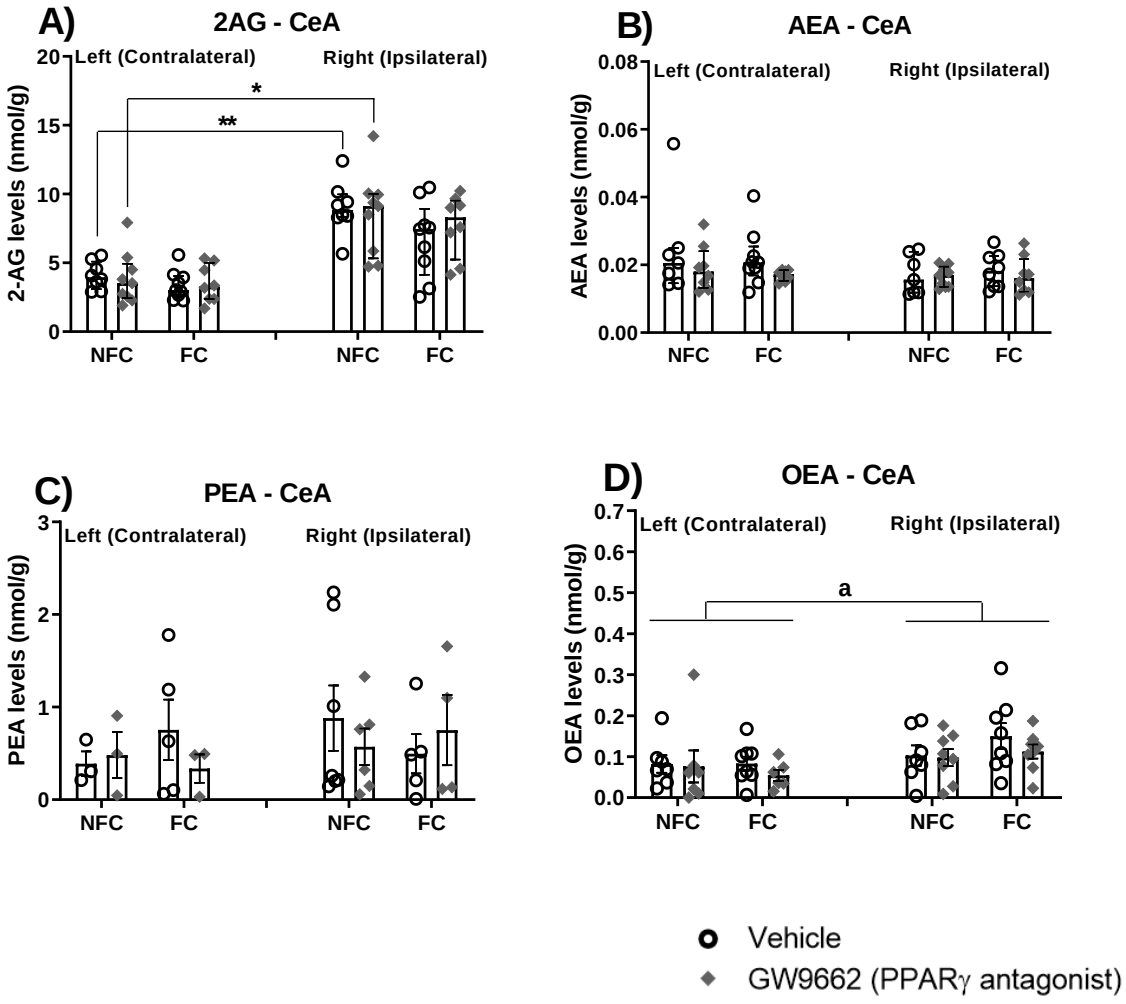


Figure 2.22: Effects of systemic administration of a selective PPARγ antagonist on 2-AG (A), AEA (B), PEA (C), and OEA (D) levels in the CeA in NFC and FC rats. Three-way ANOVA have shown a significant effect of side ($^{a}p<0.05$) on OEA levels. Post hoc analysis revealed that 2-AG levels are significantly higher in the right side in NFC groups compared to their left counterparts (**$p<0.01$ vs Left NFC Vehicle; $^{*}p<0.05$ vs Left NFC GW9662). Data are expressed as median with interquartile range (A and B) and mean ± S.E.M (C and D), n=7-9 rats per group.

2.3.2.5.3 Effect of fear conditioning and GW9662 administration on endocannabinoid and NAE levels in the VH

The analysis of the levels of 2-AG in the VH using Kruskal-Wallis comparisons revealed that there were no differences among the groups (χ^2 (7) = 10.547, $p>0.05$) (Figure

2.29A). When each side was analysed separately, Kruskal-Wallis test revealed that there were no differences among the groups in the right (Right χ^2 (3) = 3.539, p>0.05) or in the left [Left χ^2 (3) = 3.035, p>0.05] in 2-AG levels (Figure 2.17) in the VH.

The analysis of the levels of AEA in the VH using Kruskal-Wallis comparisons revealed that there were no differences among the groups (χ^2 (7) = 5.649, p>0.05) (Figure 2.29B). When each side was analysed separately, Kruskal-Wallis test revealed that there were no differences among the groups in the right (Right χ^2 (3) = 2.620, p>0.05) or in the left [Left χ^2 (3) = 0.737, p>0.05] in AEA levels in the VH.

The analysis of the levels of PEA in the VH using Kruskal-Wallis comparisons revealed that there were no differences among the groups (χ^2 (7) = 7.900, p>0.05) (Figure 2.29C). When each side was analysed separately, Kruskal-Wallis test revealed that there were no differences among the groups in the right (Right χ^2 (3) = 2.033, p>0.05) or in the left [Left χ^2 (3) = 0.839, p>0.05] in PEA levels in the VH.

The analysis of the levels of OEA in the VH using Kruskal-Wallis comparisons revealed that there were no differences among the groups (χ^2 (7) = 4.656, p>0.05) i (Figure 2.29D). When each side was analysed separately, Kruskal-Wallis test revealed that there were no differences among the groups in the right (Right χ^2 (3) = 2.544, p>0.05) or in the left [Left χ^2 (3) = 0.686, p>0.05] in OEA levels in the VH.

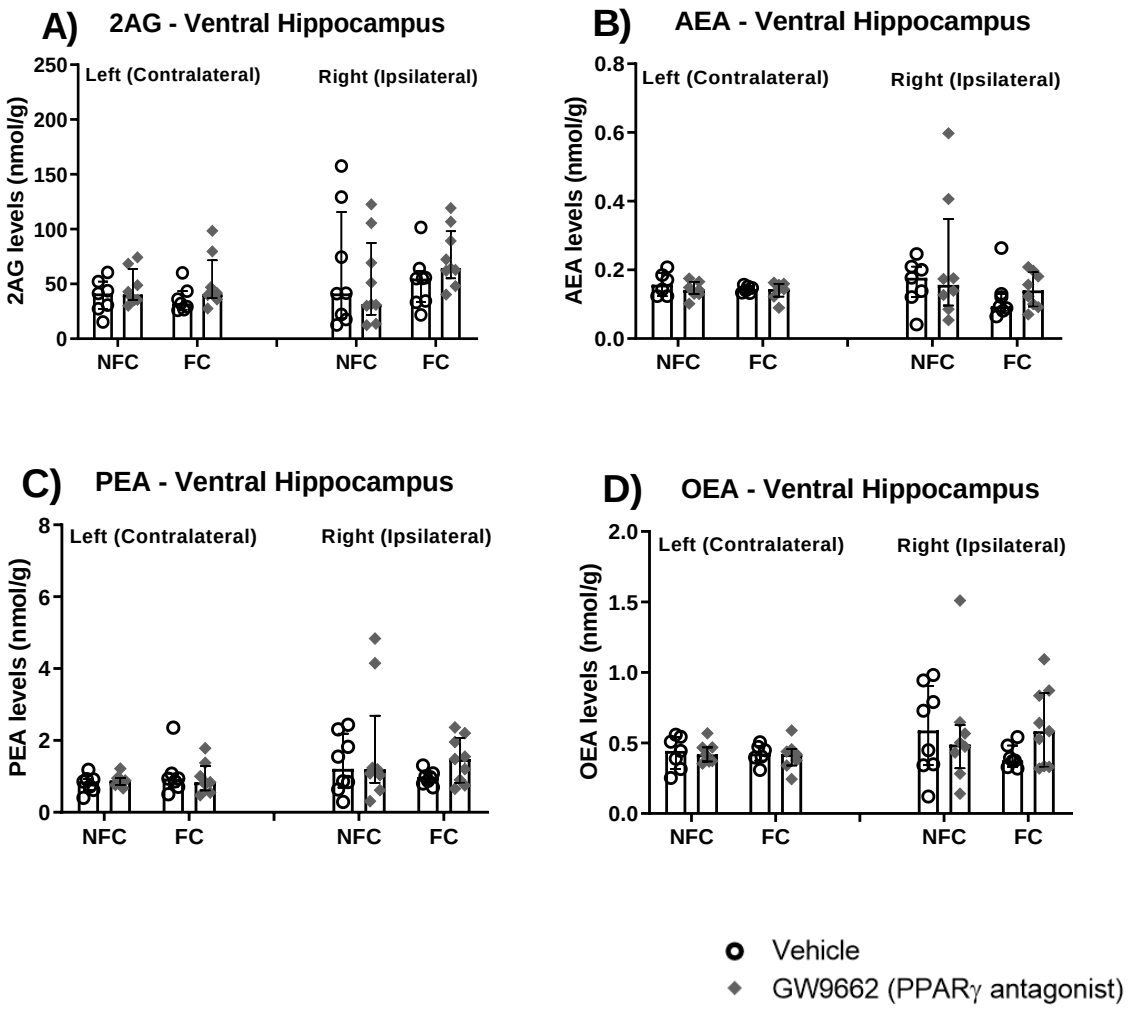


Figure 2.9: Effects of systemic administration of a selective PPARγ antagonist on 2-AG (A), AEA (B), PEA (C), and OEA (D) levels in the ventral hippocampus (VH) in non-fear conditioned (NFC) and fear conditioned (FC) rats. Data are expressed as median with interquartile range, n=7-9 rats per group.

2.4 Discussion

The two experiments described in this chapter investigated the role of PPARs in the mediation of inflammatory pain, FCA, and conditioned fear in the presence of nociceptive tone. All drugs were shown by mass spectrometry to cross the blood brain barrier and reach DH tissue. Systemic administration of the PPARα and PPARβ/δ antagonists in rats prolonged context-induced freezing in the presence of formalin-evoked nociceptive tone without affecting its initial expression, while the PPARγ antagonist potentiated freezing expression over the entire trial. These effects on fear-related behaviour were observed in the absence of any effects on formalin-evoked nociceptive behaviour or locomotor activity measured by walking duration. These novel data suggest that pharmacological blockade of PPARα and PPARβ/δ, in the presence of formalin-evoked nociceptive tone, impaired short-term, within-trial fear-extinction in rats without affecting pain response, while pharmacological blockade of PPARγ potentiated conditioned fear responding. Thus, endogenous signalling through these three PPAR isoforms may serve to reduce expression of conditioned fear in the presence of nociceptive tone. The data herein suggest a modulatory role for PPARs in fear-related behaviour.

We propose that the blockade of PPARα and PPARβ/δ delayed the short-term, within-trial extinction of fear memory without affecting initial expression of fear-related behaviour. Extinction is defined as a learned inhibition of retrieval of previously acquired memories. Therefore, the blockade of PPARα and PPARβ/δ may be impairing the formation of a new memory upon re-exposure to the conditioned arena. Most studies investigating the role of PPARs in memory have investigated their role in models of mnemonic impairment, such as diabetes-induced cognitive dysfunction (Kariharan et al., 2015; Gad et al., 2016), morphine-induced mnemonic dysfunction (Babaei et al., 2012), scopolamine-induced memory impairment (Allami et al., 2011; Almasi-Nasrabadi et al., 2014; Xu et al., 2016b), and others (Pathan et al., 2006; Liu et al., 2013; Gao et al., 2017; Kossatz et al., 2018). There are studies showing that modulation of PPARs may also affect memory formation in subjects whose mnemonic abilities were preserved. For instance, Mazzola et al. (2009) have shown that intraperitoneal administration of WY14643, a PPARα synthetic agonist, enhanced memory acquisition. Campolongo et al. (2009) have shown that post-training administration of the endogenous PPAR ligand OEA enhanced memory consolidation in both spatial and passive-avoidance learning tests, effects that were abolished in mutant mice lacking PPARα. On the other hand, Varvel et al. (2006)

demonstrated that administration of OEA and PEA before testing did not have any effect on working memory. A potential alternative explanation for our findings is that the blockade of PPARα and PPARβ/δ enhanced the recall of fearful memories, however the lack of effect of the PPARα and PPARβ/δ antagonists on the initial expression of freezing upon re-exposure to the context argues against this. In contrast, the systemic administration of the PPARγ antagonist potentiated the expression of initial freezing upon context re-exposure, and that this potentiation was maintained over the entire trial. Thus, it is possible that blockade of PPARγ enhances fear memory recall or is in itself pro-aversive (i.e. supporting an anxiolytic effect of PPARγ signalling). The latter interpretation may be more likely because previous studies demonstrated that the PPARγ activation rather than blockade improves mnemonic performance. For example, Gemma et al. (2004) have shown that the oral administration of rosiglitazone, a PPARγ agonist, improved cognitive performance in aged rats compared to young controls exposed to contextual fear conditioning. Similarly, Babaei et al. (2012) have shown that pioglitazone, another PPARγ agonist, improved the performance of mice with mnemonic impairment induced by morphine. Other studies have shown improved cognitive performance in pioglitazone-treated animals (Pathan et al., 2006; Yamanaka et al., 2012; Liu et al., 2013; Almasi-Nasrabadi et al., 2014; Gao et al., 2017). Further evidence in support of an anxiolytic effect of PPARγ signalling comes from recent work by Youssef et al. (2019) demonstrating that the administration of a PPARγ antagonist blocked the anxiolytic effect of beta-caryophyllene. Additionally, repeated stress decreased PPARγ expression in the amygdala, and treatment with buspirone or minocycline, two drugs with anxiolytic effects, recovered PPARγ expression in the same region (Liu et al., 2018). Furthermore, PPARγ blockade or knockout was shown to have anxiogenic effects on mice (Domi et al., 2016). These studies, together with the data presented here, suggest an anti-aversive/anxiolytic effect of PPARγ signalling.

The results suggest that endogenous signalling at PPARα, PPARβ/δ and PPARγ does not mediate or modulate formalin-evoked nociceptive behaviour. Our findings are in accordance with Donvito et al. (2017) who demonstrated that intraperitoneal administration of PPARα antagonist (GW6471) did not affect formalin-evoked nociceptive behaviour in mice. Previous reports have shown that systemic administration of PPARα (Taylor et al., 2002; Oliveira et al., 2007; Suardíaz et al., 2007) and PPARβ/δ (Gill et al., 2013) agonists attenuated acute inflammatory pain behaviour, which indicates an

antinociceptive effect of PPARα and PPARβ/δ activation by exogenously administered agonists (see Okine et al., 2018). However, less is known about the effects of the blockade of these receptors on inflammatory pain. To our knowledge, the current study is the first to investigate the effects of the blockade of PPARβ/δ on inflammatory pain. Previous studies have shown that systemic administration of pioglitazone, a widely used PPARγ agonist, attenuates formalin-induced nociceptive response (Oliveira et al., 2007; Mansouri et al., 2017b). In their study, Mansouri et al. (2017) also indicated that systemic administration of GW9662 alone did not have any effect on nociceptive behaviour, which is in line with our findings.

FCA is a potent suppression of nociceptive responses upon exposure to a fearful stimulus. It has been shown to be associated with increased levels of AEA, an endocannabinoid which also binds to PPARs, in the basolateral amygdala (BLA) (Rea et al., 2013b) and in the dorsolateral periaqueductal grey (dlPAG) (Olango et al., 2012) and a strong trend for increased tissue levels of PEA and OEA, endogenous ligands of PPARs, in the BLA (Fu et al., 2003a; LoVerme et al., 2005). The experiments described in this chapter investigated the effects of administration of PPAR antagonists on FCA. The data demonstrate that fear conditioning profoundly reduces formalin-evoked nociceptive behaviour via FCA as we and others have shown previously (Roche et al., 2010; Ford et al., 2011; Rea et al., 2011, 2014; Butler et al., 2012; Olango et al., 2012) and that the blockade of PPARα, PPARβ/δ or PPARγ does not affect expression of FCA. However, a limitation of the present experiments is that the trial duration (15 minutes) was short and, consequently, restricts an analysis of possible alterations in FCA at later time points beyond the initial 15min period where FCA is very robust. Specifically, an enhancement of FCA by PPARs blockade would have been very difficult to observe due to the minimal expression of nociceptive behaviour in FC rats during this initial 15min period. Hence, future investigations using an extended trial duration could further explore the role of these receptors in FCA.

The LC-MS/MS analysis suggests that the prolongation of freezing in the presence of nociceptive tone upon the administration of PPARα and PPARβ/δ antagonists was associated with increased levels of GABA and glutamate in the BLA and ventral hippocampus, with ANOVA revealing an overall drug treatment effect for both neurotransmitters in these regions, although *post hoc* analysis did not reach significance,. Moreover, GW6471 and GSK0660 also increased levels of dopamine in the right CeA of

FC animals only. Increased GABAergic transmission before extinction training impairs extinction retention (see Makkar et al (2010) for a review on the role of GABA in learning and memory) and the acquisition of an extinction memory is related to an upregulation of different GABA-related genes (Orsini and Maren, 2012). A study from Sasso et al (2010) has shown that intracerebroventricular (i.c.v.) injection of PEA leads to a increased activation of $GABA_A$ receptors through PPARα, showing that these two systems may interact. We suggest that the blockade of PPARα and PPARβ/δ increases levels of GABA in the BLA, possibly leading to an impairment of extinction learning in the test session. The formation of extinction memories is dependent of NMDA glutamatergic receptors. Several studies have shown that NMDA receptor antagonism blocks or impairs fear extinction (Orsini and Maren, 2012). Therefore, the higher levels of glutamate in PPARα and PPARβ/δ treated groups may be the result of a compensatory effect caused by the GABA interference described above. Although the role of the dopaminergic signalling in the amygdala (Guarraci et al., 1999; Pezze et al., 2005), especially in the BLA (Shi et al., 2017) , is well known, the role of this neurotransmitter in the CeA in fear conditioning is less understood. The administration of D_2 antagonist into the amygdala (mainly targeting the BLA) lead to learning deficits in protocols of classical fear conditioning and fear-potentiated startle (Guarraci et al., 2000; Greba et al., 2001). Studies suggest that PPARs modulate dopamine signalling. The treatment with WY14643 (PPARα agonist) injected into the hypothalamus was shown to increase dopamine in the nucleus accumbens (Mijangos-Moreno et al., 2016c). Two other PPARα agonists, which were systemically administered, dose-dependently decreased nicotine-induced excitation of dopamine neurons in the VTA and nicotine-induced elevations of dopamine levels in the nucleus accumbens shell of rats (Mascia et al., 2011). Thus, we hypothesize that the blockade of PPARα and PPARβ/δ in FC rats increases dopamine release in the CeA. This enhancement in dopamine levels in the CeA may lead to a deficit in extinction learning, but does not appear to be involved in fear acquisition/recall since there was no alteration in dopamine levels in GW9662-treated animals. Further studies are required to address the molecular mechanisms behind this effect. LC-MS/MS analysis did not indicate any changes in levels of endocannabinoids or NAEs associated with administration of PPARα, PPARβ/δ and PPARγ antagonists in FC or NFC rats in the BLA, CeA or VH. These results suggest that the effects on fear behaviour upon administration of GW6471, GSK0660 and GW9662 related above occur in the absence of changes in endogenous PPAR ligand levels.

In conclusion, these studies have shown that the systemic administration of PPARα and PPARβ/δ antagonists impaired short-term, within-trial fear-extinction in rats without affecting pain response and in the presence of a nociceptive stimulus. Likewise, the systemic administration of the PPARγ antagonist potentiated freezing expression in the presence of a nociceptive tone. These results indicate a possible modulatory role for PPARs in fear/anxiety expression in the presence of pain, but further investigations are necessary to elucidate the possible molecular mechanisms and neural substrates involved in this modulation. In order to explore this possible role PPARs in fear/cognition and anxiety, we conducted five further studies – chapters 3 and 4 explore the role of PPARs expressed in the BLA and CeA in conditioned fear in the presence versus absence of pain, and chapter 5 examines the role of PPARs in innate anxiety and cognition in the presence and absence of pain.

Chapter 3: Effects of intra-BLA administration of PPAR antagonists on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence or absence of nociceptive tone in rats

3.1 Introduction

PPARs are transcription factors and part of the nuclear hormone superfamily of receptors. There are three described isoforms: PPARα, PPARβ/δ and PPARγ (Issemann and Green, 1990). Endogenous ligands at PPARs, include fatty acids (Marion-Letellier et al., 2016), serotonin derivatives (Waku et al., 2010a), and NAEs including AEA (Bouaboula et al., 2005; Rockwell et al., 2006), PEA (LoVerme et al., 2005) and OEA (Fu et al., 2003). PPARs are involved in many physiological processes and are targets for current in-use medicines for diabetes (Hong et al., 2018) and cholesterol lowering (Fruchart et al., 2001).

The amygdala is part of the limbic system and plays a key role in emotional responses including anxiety and fear (Davis, 1992). According to the nomenclature proposed by Price, the BLA is one of three groups of nuclei in the amygdala. It differentiates itself from the other two groups - CeA and cortical nuclei – on account of its connections, embryonic origin and cytoarchitecture (Sah et al., 2003). The BLA receives input from several brain regions, including the hippocampus, hypothalamus, cerebral cortex, ventral tegmental area (VTA), and thalamus (Sah et al., 2003; LeDoux, 2007; Tsvetkov et al., 2015). It also has an extensive efferent network, which includes the CeA, PAG, ventral striatum, dorsal striatum (caudate-putamen), hippocampus, and others (Sah et al., 2003; LeDoux, 2007; Tsvetkov et al., 2015). The BLA has a central role in fear conditioning. It has been shown that lesions (Helmstetter, 1992a; Phillips and LeDoux, 1992; Sananes and Davis, 1992; Kim et al., 1993; Maren, 1993, 1999; Koo et al., 2004) or inactivation by muscimol (Miserendino et al., 1990; Fanselow and Kim, 1994; Helmstetter and Bellgowan, 1994; Muller et al., 1997; Sacchetti et al., 1999) of the BLA impaired acquisition and expression of fear conditioning. Inactivation of the BLA also affects fear extinction (Baldi and Bucherelli, 2010). The GABAergic (Makkar et al., 2010a), glutamatergic (Davis and Myers, 2002; Walker and Davis, 2002a), serotoninergic (Bauer, 2015a), dopaminergic (Fadok et al., 2010), and endocannabinoid (Chhatwal and Ressler, 2007) systems were shown to participate in this modulatory role of the BLA in fear and anxiety processing.

Pain is a complex condition with sensory-motor, emotional and cognitive aspects. The amygdala is part of both the descending pain pathway and the limbic system and is involved in the emotional-affective aspect of pain. The BLA was shown to be important in pain processing. Neurons in the BLA respond to chronic (Ji et al., 2010) and acute (Luongo et al., 2013; Butler et al., 2017) noxious stimuli and the pharmacological deactivation of the BLA reduced pain-related behaviour (Ji et al., 2010). Additionally, intra-plantar injection of formalin increased *c-fos* expression in the BLA (Nakagawa et al., 2003).

Pain and fear modulate one another in a reciprocal manner. The phenomenon known as fear-conditioned analgesia (FCA), in which a fearful stimulus causes a significant suppression in pain response, is an example of the influence of fear on pain. In turn, pain can regulate fear responses. Post-traumatic stress disorder (PTSD) symptoms tend to be more pronounced in patients with chronic pain (Asmundson et al., 2002). Moreover, patients with chronic pain are twice as likely to develop phobias (Pereira et al., 2017). PPAR isoforms are expressed in brain regions that play an important role in pain and fear/anxiety such as the amygdala (Warden et al., 2016), PFC (Moreno et al., 2004; Okine et al., 2014; Warden et al., 2016), hippocampus (Moreno et al., 2004; Domi et al., 2016) and PAG (Okine et al., 2017).

Studies have indicated a likely role for PPARs in pain (see Okine et al., 2018 for review), but the role of PPARs expressed in the amygdala in pain has not yet been examined. There is some evidence that PPARγ blockade or knockout has anxiogenic effects in mice (Domi et al., 2016; Youssef et al., 2019). However, whether PPARβ/δ modulate anxiety or fear remains unexplored. Moreover, the role of PPARs expressed in the BLA in interactions between pain and fear has not yet been investigated. The studies described in the previous Chapter 2 provided evidence that PPAR blockade can potentiate conditioned fear-related behaviour in the presence of nociceptive tone but the brain regions mediating these effects remain to be elucidated. In that context, the present chapter focused on the role of PPARs in the BLA in pain, fear and FCA.

In this chapter, I investigated the hypothesis that the blockade of PPARs expressed in the BLA enhances FCA, increase conditioned fear, and decreases tonic inflammatory pain. Specifically, I examined the effects of intra-BLA administration of GW6471 (PPARα antagonist), GSK0660 (PPARβ/δ antagonist), and GW9662 (PPARγ antagonist) on formalin-induced nociceptive behaviour and FCA in rats. I also investigated the effects of

intra-BLA administration of these antagonists on conditioned-fear related behaviour both in the presence and absence of nociceptive tone in rats. In addition, associated alterations in levels of neurotransmitters, endocannabinoids, and NAEs in the BLA were analysed. Furthermore, differences in the levels of neurotransmitters, endocannabinoids and NAEs in FC and NFC rats that received either formalin or saline injection were also analysed. Therefore, the specific aims of the studies described in this chapter were:

- To verify the expression of PPARα, PPARβ/δ and PPARγ in the rat BLA by Western Blotting or RT-qPCR.
- To determine if PPAR signalling within the BLA plays a role in tonic persistent inflammatory pain and FCA by examining the effects of intra-BLA administration of PPAR antagonists on formalin-evoked nociceptive behaviour and FCA in rats, and associated alterations in levels of neurotransmitters, endocannabinoids, and NAEs in the BLA.
- To determine if PPAR signalling within the BLA plays a role in expression of conditioned fear in the presence and in the absence of nociceptive tone by examining the effects of intra-BLA administration of PPAR antagonists on fear-related behaviour, and associated alterations in levels of neurotransmitters, endocannabinoids, and NAEs in the CeA.
- To determine if the presence of nociceptive tone influences the levels of neurotransmitters, endocannabinoids and NAEs in NFC and FC rats

3.2. Materials and Methods

3.2.1 Animals

Experiments were carried out on a total of 88 (Experiment 1) and 92 (Experiment 2) adult male Sprague-Dawley rats (230-250g on arrival; Envigo UK, Bicester, England). The animals were maintained at controlled temperature (22 ± 2^{o}C) and humidity (45-55%) under standard lighting conditions (12:12h light-dark cycles, lights on from 07.00h). Animals were housed 2-3 per flat bottomed cage (L:45 x H:20 x W:20cm) containing 3Rs paper bedding material (Fibrecycle Ltd., North Lincolnshire, United Kingdom) and sizzle nest material (LBS Biotechnology, Horley, United Kingdom) for the first week after arrival, and were posteriorly singly housed after surgery and for the rest of the experiment. Food (14% Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, 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.

3.2.2 Cannula Implantation

Under isoflurane anaesthesia (2-3% in O_2, 0.7L/min), a stainless steel guide cannula (12mm length, Plastics One Inc., Roanoke, Virginia, USA) was stereotaxically implanted 1mm above the right and left BLA of each rat (coordinates: AP = -2.5 mm from bregma, ML = ±4.8 mm, DV = -7.5 mm from the skull surface) according to the rat brain atlas published by Paxinos and Watson, 1997. The cannulae were permanently fixed to the skull using stainless steel screws and carboxylate cement. A stylet made from stainless steel tubing (12mm length, 22G, Plastic One – Bilaney Consultants, Sevenoaks, UK) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (1.25mg/25µL, s.c., Rimadyl, Pfizer, Kent, UK), was administered before the surgery to manage postoperative analgesia. Animals received a single daily dose of the antimicrobial agent enrofloxacin (10mg/kg, s.c., Baytril, Bayer plc, Berkshire, UK) for 5 days to prevent postoperative infection. Following cannula implantation, the rats were singly housed and at least 6 days were allowed for recovery post-surgery prior to experimentation. During this recovery period, the rats were handled, stylets checked, and their body weight and general health monitored once daily.

3.2.3 Drugs

PPARα antagonist, GW6471, PPARβ/δ antagonist, GSK0660, and PPARγ antagonist, GW9662 (all obtained from Tocris Bioscience, Bristol, UK) were dissolved in a 100% Dimethyl sulfoxide (DMSO), used as vehicle solution. The dose of GW6471 (10μg/0.5μl) was chosen based on a study from our laboratory showing that this dose delayed the onset of the second phase of formalin-evoked nociceptive behaviour (Okine et al., 2004). The dose of GW9662 (10μg/0.5μl) was chosen based on a previous study showing that this dose was effective in reversing the anti-inflammatory and anti-hyperalgesic actions of rosiglitazone (Morgenweck et al., 2010). We used the same dose of GSK0660 (10μg/0.5μl) as that used for the other two antagonists for comparison and because no published studies have administered this drug intracerebrally. Formalin was prepared from a 37% stock solution (Sigma-Aldrich, Dublin, Ireland) diluted in sterile saline. Sodium chloride was dissolved in distilled water (9g in 1L – 0.9%) and the solution was autoclaved.

3.2.4 Experimental Procedure

Two different experiments using two different cohorts of rats were carried (Experiments 1 and 2) and were identical in design and methodology with the exception that rats in Experiment 1 received intra-plantar injection of formalin while those in Experiment 2 received intra-plantar injection of saline. The FCA paradigm used in both experiments was essentially as described before (Finn et al., 2004; Butler et al., 2008; Rea et al., 2018) and in Chapter 2 (Section 2.2.1). There were two phases: conditioning (day 1) and test (day 2). On the conditioning day, rats were placed in a Perspex chamber (30 cm x 30 cm x 40 cm) and after 15 seconds they received the first of 10 footshocks (0.4mA, 1 second duration, LE85XCT Programmer and Scrambled Shock Generator; Linton Instrumentation, Norfolk, UK) spaced 60 seconds apart. Fifteen seconds after the last footshock, rats were returned to their home cage. The animals that belonged to the control group, that did not receive footshocks, were placed in the chamber for an equivalent time (9min 30s). The animals were randomly assigned to one of 8 groups (n = 11 per group) – rats that received footshocks (FC) or no footshocks (NFC) treated with the PPARα antagonist GW6471, the PPARβ/δ antagonist GSK0660, the PPARγ GW9662, or vehicle (100% DMSO). The

sequence of testing was randomized to minimize any confounding effects of the order of testing.

The test day started 23hrs 30min after the end of the conditioning phase (Figure 1). First, the rats received a 50µl injection of formalin (2.5% in saline; Experiment 1) or saline (Experiment 2) into the right hind paw under brief isoflurane anaesthesia (3% in O_2; $0.8L \cdot min^{-1}$). Fifteen minutes later, the animals received intra-basolateral amygdalar (intra-BLA) microinjections of either the PPARα antagonist (GW6471), the PPARβ/δ antagonist (GSK0660), PPARγ antagonist (GW9662) or vehicle (volume of injection 0.5 µl/side). After these microinjections, the rats were returned to their home cages. Fifteen minutes after microinjections, or 24 hours after footshock, the rats were re-exposed to the conditioning chamber. A video camera located beneath the observation chamber was used to monitor animal behaviour for 30 min. For this experiment, it was decided that 30 minutes duration re-exposure was more adequate to observe changes in FCA than the 15 minutes used in the first chapter. At the end of the test phase (60 min post formalin injection), rats were killed by decapitation, fast-green dye injected via the guide cannulae (see below), brains were removed, snap-frozen on dry ice and stored at −80°C. Formalin induced oedema was assessed by measuring the change in the diameter of the right hind paw measured immediately before, and 60 min after, formalin administration, using Vernier callipers.

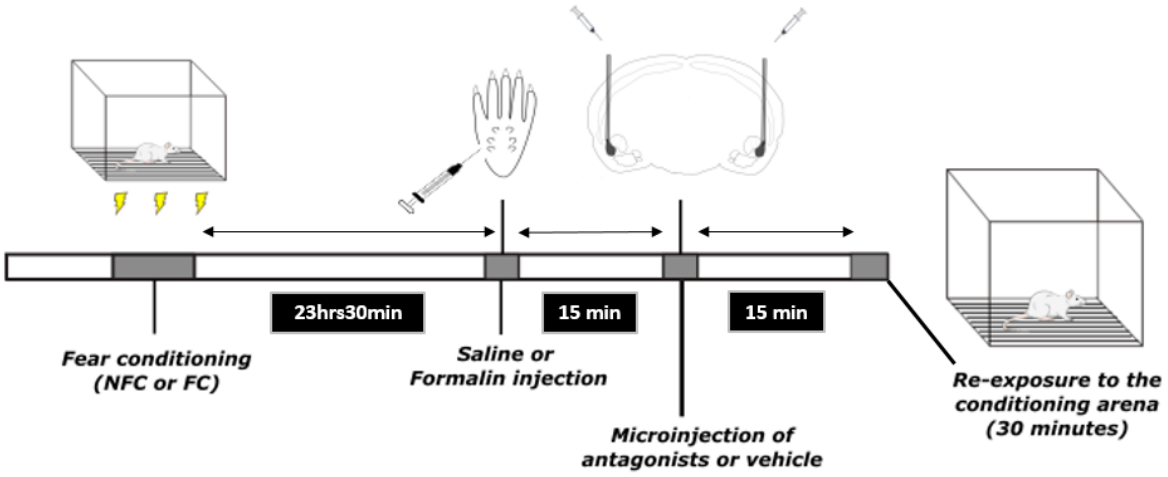


Figure 3.1: Graphical representation of the experimental procedure.

Experiments	Intraplantar injection	Treatment	Conditioning	
			NFC (n per group)	FC (n per group)
Experiment 1	Formalin	Vehicle	11	11
	Formalin	GW6471 (PPARα antagonist)	11	11
	Formalin	GSK0660 (PPARβ/δ antagonist)	11	11
	Formalin	GW9662 (PPARγ antagonist)	11	11
Experiment 2	Saline	Vehicle	11	11
	Saline	GW6471 (PPARα antagonist)	11	11
	Saline	GSK0660 (PPARβ/δ antagonist)	11	11
	Saline	GW9662 (PPARγ antagonist)	11	11

Table 3.1: Summary of experimental groups. NFC, non-fear conditioned; FC, fear conditioned.

3.2.5 Behavioural analysis

See Chapter 2, section 2.2.4

3.2.6 Brain extraction

See Chapter 2, section 2.2.5

3.2.7 Histological verification of intracerebral injection sites

Stereotaxic coordinates were verified histologically on 2 animals before the start of the cannula implantation surgeries. The rats underwent the surgical procedure detailed in the section 3.2.2. After the conclusion of the surgical implantation of cannulae, the 2 rats, still under anaesthesia, were decapitated and a microinjection of 2% fast green dye (0.5 μl over 1 minute; Sigma-Aldrich, Dublin, Ireland) diluted in DMSO was made to determine if the coordinates used were accurate for the BLA. The brains were collected and snap-frozen on dry ice. Then, frozen coronal brain sections were cut at 50 μm thickness on a cryostat at -

21°C from the start to the end of the amygdaloid complex to determine the location of the dye and confirm coordinates. For all other rats in the experiments, the dye injections were performed immediately post-decapitation in order to determine if the injections successfully targeted the BLA.

3.2.8 Cryo-sectioning and tissue microdissection

Frozen coronal brain sections of 150 µm thickness containing the basolateral amygdala (BLA) were cut on a cryostat (Leica Biosystems, Watznal, Germany), and were punch-dissected as previously described (Ford et al., 2008; Olango et al., 2012a; Rea et al., 2014) using cylindrical brain punchers (Harvard Apparatus, MA, USA) with an internal diameter of 0.50 mm for the different amygdalar nuclei, at the following rostro-caudal levels (obtained from the rat brain atlas by Paxinos and Watson, 1997: (BLA) Bregma, - 2.12 – -3.30 mm. Additionally, in order to evaluate possible lateralisation effects, the BLA punches were separately collected for right and left hemispheres. The punch-dissected tissue was weighed (mean ± S.E.M. weight per sample was 1.72 ± 0.1 mg) and stored at -80°C prior to measurement of AEA, PEA, OEA, 2-AG and neurotransmitter levels by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

3.2.8 Measurement of endocannabinoids, NAEs and neurotransmitters in discrete brain regions using liquid chromatography – tandem mass spectrometry (LC-MS/MS)

See Chapter 2, section 2.2.7

3.2.9 Verification of PPAR expression in the BLA

3.2.9.1 Verification of PPAR expression in the BLA by Western blotting

Punched brain tissues from BLA of naïve male SD rats were analysed by western immunoblotting. Frozen punched samples were lysed by brief 3s sonication in radio-immunoprecipitation assay (RIPA) lysis buffer (150mmol/L NaCl, 25mmol/L Tris-HCl, pH 7.6, 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1mmol/L Na_3VO_4, 10mmol/L NaF containing 1% protease inhibitor cocktail [Sigma-Aldrich, Ireland] in a 1.5mL microcentrifuge tube [75 ml]). After homogenisation, the microcentrifuge tube was placed on the shaker for 45 minutes as 4°C for the RIPA lysis buffer to free the protein bound either to plasma membrane or nuclear membrane and then centrifuged at 14000g (Eppendorf Centrifuge 5415R Stevenage, UK) for 20min at 4°C to separate the precipitate and the supernatant. The supernatant was collected and protein content determined by

Bradford assay. Protein (BSA, Sigma-Aldrich, Ireland) standards (0, 0.0125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 mg/ml) were prepared in deionised water (DH_2O). The Bradford assay involved adding 250 µl Bradford reagent (Sigma-Aldrich, Ireland) to 5µl of unknown samples or standards in triplicate on a 96-well plate. After a 5min incubation time, absorption at 570nm wavelength was determined. Protein concentrations of the samples were determined using 8 point standard curve constructed using the BSA standards. The samples were equalised to 2.0mg/ml after determining the protein concentration. 8µl of 4X sample loading buffer was added to 24µl of protein sample (48µg of protein sample) in the microcentrifuge tubes (4X sample loading buffer: 25% v/v 1 mol/L Tris-HCl, pH 6.8, 5% w/v sodium dodecyl sulphate (SDS), 20% v/v glycerol, 2.5% Bromophenol blue (0.2% w/v in 100% ethanol), 7M Urea, and 20% v/v of 2-mercaptoethanol, made up to a total volume of 20mL in distilled water). The microcentrifuge tubes were vortexed quickly and then boiled at 95°C for 5mins. The samples then are briefly centrifuged and subjected to 9% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 120 mV for 2 hrs. The separated protein samples were electroblotted onto a nitrocellulose membrane (Nitrocellulose membrane, CAS# 9004-70-0; Bio-Rad, Ireland) at 100mV for 40 min using wet transfer method. Protein transfer efficiency was verified by ponceau S (0.1% ponceau dye in 5% acetic acid; Sigma-Aldrich, Ireland) staining of protein band. Membranes were blocked in 5% non-fat dry milk in 0.1% Tris-buffered saline/Tween 20 (TBST) solution for 1hr at room temperature and incubated with a polyclonal antibody to PPARα [1:200, Cat# 398394, anti-rabbit, SantaCruz Biotechnology, USA], PPARβ/δ [1:200, Cat# 74517, anti-mouse SantaCruz Biotechnology, USA] or PPARγ receptor [1:200, Cat# 22020, anti-goat, SantaCruz Biotechnology, USA] and mouse monoclonal antibody to β-actin (1:10000 Cat# 5441; Sigma-Aldrich, Ireland) diluted in 5% milk/0.05% TBST overnight at 4°C. Post incubation period, the membrane was washed in washing buffer (0.1% TBST) for 3 x 10 min washes. After the washing, membranes were then incubated in secondary antibody solution containing IR-Dye goat anti-mouse (k700) and goat anti-rabbit or donkey anti-goat (k800) (LI-COR Biosciences, UK) diluted 1:10,000 in 1% milk/0.1% TBST for 1hr. Five x 5min washing steps were then performed with washing buffer (0.1% TBST) and 1 final 5min wash in distilled water. Blots were scanned on a LI-COR Odyssey imager. IR band intensities for PPARs receptor protein expression (~52/55kDa) for each sample were generated automatically using the background subtraction method of the LI-COR Image Studio Ver. 2.0 imaging software. Two distinct bands were observed for PPARγ (Refer to

figure 3.19C), due to the existence of two isoforms for this receptor. Because the antibodies for PPARα and PPARβ/δ were raised in mouse, similarly to the β-actin, these two isoform had the band for the endogenous control taken in a second moment. The membranes were stripped of the binding of PPAR antibodies using a stripping buffer (Appendix B), and the protocol described above was repeated from the blocking in 5% non-fat dry milk in 0.1% TBST step, and the membrane was then re-probed using β-actin antibodies. The blots were then re-scanned on a LI-COR Odyssey imager. IR band intensities for β-actin (~42kDa) were generated automatically using the background subtraction method of the LI-COR Image Studio Ver. 2.0 imaging software. Full details of the composition of all buffers/solutions used are provided in Appendix B.

3.2.9.2 Verification of PPARβ/δ expression in the BLA by RT-qPCR

Punched brain tissues from right and left BLA of four male naïve Sprague Dawley rats were analysed by quantitative real-time PCR (RT-qPCR). RT-qPCR was carried out as described previously (Burke et al., 2014, Kerr et al., 2012, Rea et al., 2014). RNA was extracted from BLA tissue (BLA: 2.04mg ± 0.2mg) using the Macherey-Nagel NucleoSpin ® RNA extraction kit (Nucleospin RNA, Fisher Scientific, Ireland), according to the instructions of the manufacturer. Tissue was homogenised in 353.5µl of lysis buffer (RA1) containing β-mercaptoethanol (Sigma, Ireland) for 3-5s using an automated homogenizer (Polytron tissue disrupter, Ultra-Turrax, Germany). Homogenates were kept on ice until transferred to a Nucleospin filter (violet ring) and centrifuged at 11000g for 1 min to reduce viscosity and clear the lysate. The lysates were then treated with 350µl of 70% molecular grade ethanol (Sigma, Dublin, Ireland) and transferred to a Nucleospin RNA column (light blue ring) and centrifuged at 11000g for 30s to bind the RNA to the membrane. The membrane column was then desalted by adding 350µl membrane desalting buffer (MDB) and centrifuging at 11000g for 1 min to dry the membrane. Samples were then treated with 10µl rDNase and left for 15 minutes at room temperature to remove any DNA. Samples were then serially washed using washing buffers (200µl RA2, 600µl RA3 and 250µl RA3) and RNA was eluted in 30µl of RNAase-free water (Sigma, Dublin, Ireland). Nanodrop technology (ND-1000, Nanodrop, Labtech International, Ringmer, UK) was used to measure the concentration, purity and integrity of the RNA. RNA concentration was determined by measuring optical density (OD) at 260 nm. The integrity and purity were determined by measuring the ratios OD260/OD280 and OD230/OD280, respectively, where a ratio of

approximately 1.8-2.0 was deemed indicative of RNA of good quality and purity. All RNA samples were within the acceptable range for both integrity and purity. Samples were equalised to the same concentration of RNA (35ng/µl) using RNase free water (Sigma, Ireland). Equalised samples were then stored at -80ºC until reverse transcribed. Equal amounts of total RNA (10ng/µl) were reverse transcribed into cDNA as follows: Two master mixes were made up as shown below in Tables 5.3 and 5.4; all reagents were obtained from (Biosciences, Dublin, Ireland). 10µl of normalised RNA from each sample was added to a newly labelled PCR tube where 2µl of master mix 1 was added to each tube. The mixture was then heated to 65ºC for 5 minutes in a thermocycler (MJ Research, Reno, USA) and quickly chilled on ice. The contents of the tube were collected by brief centrifugation. 7µl of master mix 2 was then added to each tube and incubated at 37ºC for 2 minutes on the thermocycler. 1µl of superscript III reverse transcriptase was added to each sample and mixed gently. Samples were left to incubate at room temperature for 10-minutes and then loaded on the thermocycler to incubate further at 50ºC for 50 minutes. The reaction was inactivated by heating the samples at 70ºC for another 15 minutes. Finally, cDNA samples were diluted (1:4) using RNAase-free water and stored at -20ºC.

Table 3.2: Master mixture 1 for cDNA synthesis

Reagents	**Per Sample**
Random Primers (250ng)	1µl
10mm dNTP mix	1µl
Total	**2µl**

Table 3.3: Master mixture 2 for cDNA synthesis

Reagents	**Per Sample**
5X First Strand Buffer	4µl
0.1M DTT	2µl
RNase Out	1µl
Total	**7µl**

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 a FAM-labelled TaqMan probe were used (Bio-Sciences, Dublin, Ireland). Assay IDs for the genes in rats examined were as follows: PPARβ/δ (Rn00565707), and VIC-labelled β-actin (Rn00667869_m1) was used as the house keeping gene and endogenous control. A reaction

mixture was prepared and stored on ice. This consisted of 0.5µl target (PPAR) primers (Bio-Sciences, Dublin, Ireland), 0.5µl of the reference gene β-actin, 5µl TaqMan Universal PCR master mix, 1.5µl of RNA free water and 2.5µl of sample cDNA to give a total volume of 10µl per sample. Samples were pipetted in duplicate (10µl per well total volume) into an optical 96 well plate. Negative controls were included in all assays, containing the master mix but cDNA was replaced with RNase free water. Plates were then covered with adhesive covers and spun at 1000g for 1 minute to ensure complete mixing. The plate was then placed in StepOnePlus™ real time PCR machine (Bio-Sciences, Dublin, Ireland). StepOnePlus™ cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of (95°C for 15 sec/60°C for 1 min). Amplification plots were examined using Applied Biosystems 7500 System SDS Software 1.3.1.

3.2.10 Statistical Analysis

The SPSS 21.0 statistical package was used to analyse data. Normality was assessed using Shapiro-Wilk test and homogeneity of variance was checked using Levene's test. Behavioural data were analysed using two-factor analysis of variance (Two-way ANOVA), with factors being fear-conditioning and treatment, or analysis of variance with repeated measures (repeated measures ANOVA) when appropriate (e.g. when the data were analysed and presented in time bins). Neurochemical data were analysed using three-factor analysis of variance (Three-way ANOVA), with factors being fear conditioning, treatment, and side (ipsilateral or contralateral, with respect to the formalin injection). *Post hoc* pairwise comparisons were made with Student Newman-Keuls test when appropriate. If data were found to be non-parametric, three transformation were applied, in this order: square root of the data values, log of the data values, and ranking of the data values. Also, it was checked if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular data set being analysed (Thunder et al., 2007). If data were still deemed non-parametric after these transformations and tests, they were analysed using Kruskal-Wallis analysis of variance and *post hoc* analysis performed using Dunn's test when appropriate. When repeated measures data were non-parametric they were analysed using Friedman's and Kruskal Wallis tests followed by Dunn's *post hoc* if applicable. Data were considered significant when $p<0.05$. Data are expressed as group means ± standard error of the mean (S.E.M.) when parametric and as median with interquartile range when non-parametric.

Possible presence of outliers was checked by assessing the distribution of data. In case the data 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.

3.3 Results

3.3.1 Experiment 1: Effects of intra-BLA administration of PPAR antagonists on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats

3.3.1.1 Histological verification of microinjection sites

After histological verification, 75% of the rats had both injections correctly placed within the borders of both BLA. Also, 4% had one of the injections in the BLA and the other outside BLA borders. The remaining 21% were placed in the CeA, basomedial amygdala (BMA), or ventral endopiriform nucleus. The data analysed were derived only from rats where intracerebral microinjections were accurately placed in the BLA.

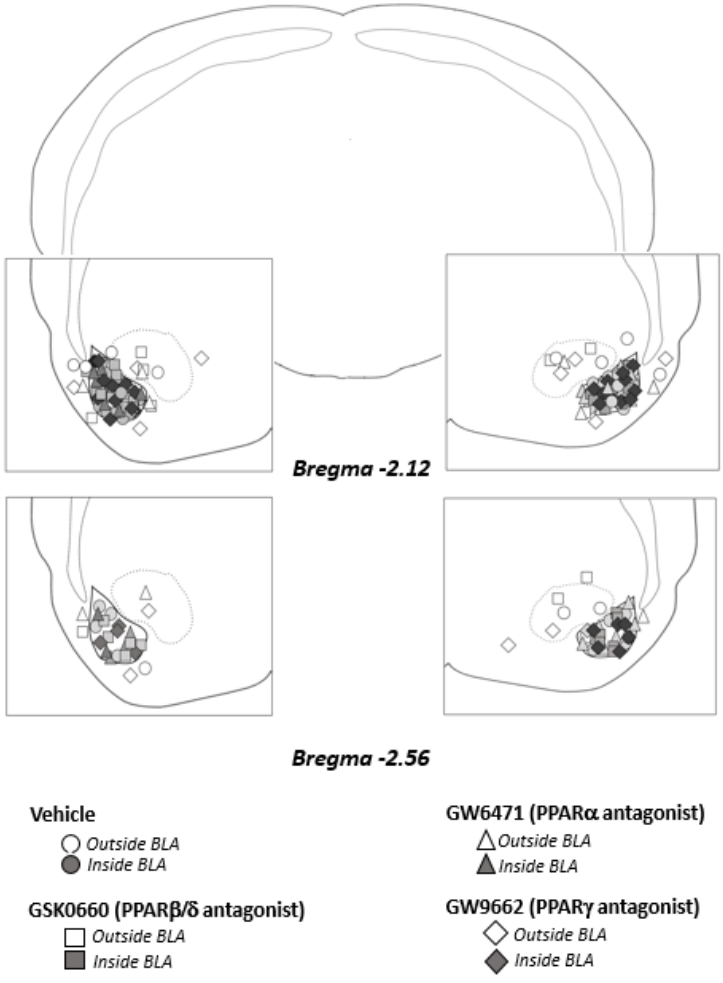


Figure 3.2: Histological verification of injector site location.

3.3.1.2 Intra-BLA administration of GW6471, GSK0660, and GW9662 had no effect on formalin-evoked nociceptive behaviour or FCA

Intra-plantar administration of formalin into the right hind paw produced robust nociceptive behaviour as evidenced by the CPS (Figure 3.3). Two-way ANOVA revealed a significant main effect of fear conditioning [$F(1, 54) = 35.264$, $^{a}p<0.05$], but not of treatment [$F(3, 47) = 0.987$, $p>0.05$] or treatment x conditioning [$F(2, 54) = 0.304$, $p>0.05$], on nociceptive behaviour (Figure 3.2). However, *post hoc* pairwise analysis with Student Newman-Keuls test did not reveal significant differences between groups. There were no significant effects of fear-conditioning [$F(1, 70) = 0.011$, $p>0.05$], treatment [$F(3, 70) = 0.296$, $p>0.05$], or treatment x conditioning [$F(2, 70) = 0.078$, $p>0.05$] on formalin-induced paw oedema (Figure 3.4).

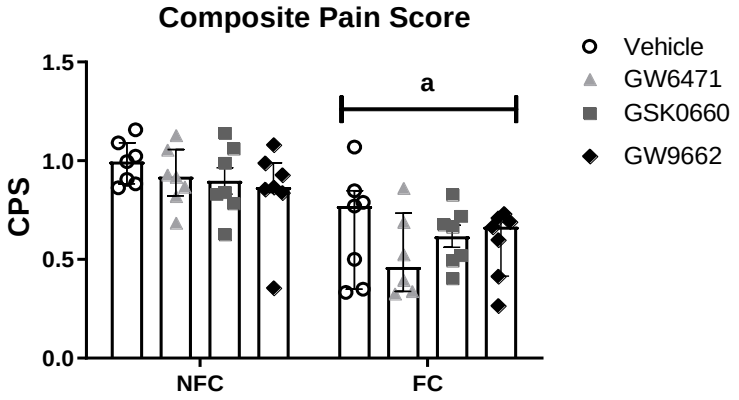


Figure 3.3: Effects of intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on formalin-evoked nociceptive behaviour in non-fear conditioned (NFC) and fear conditioned (FC) rats. Data are expressed as mean ± S.E.M (n=8-10 rats per group). According to a 2-way ANOVA ($^{a}p<0.001$), significant overall effect of fear conditioning.

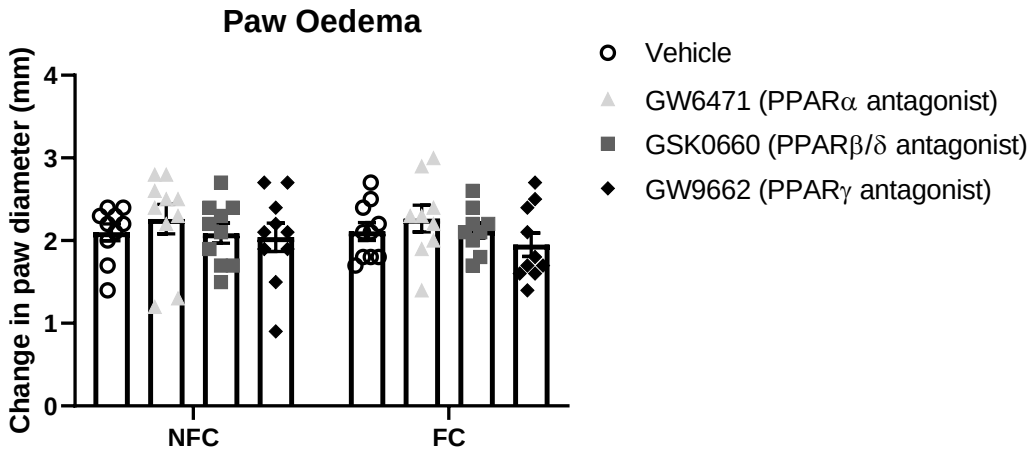


Figure 3.4: Effects of intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on paw oedema. Paw oedema was assessed by measuring the change in the diameter of the right hind paw immediately before, and 60min after, formalin administration. Data are expressed as mean ± S.E.M, n=7-9 rats per group.

3.3.1.3 Intra-BLA administration of GW6471, GSK0660, and GW9662 increases fear-related behaviour in formalin-treated rats

Kruskal-Wallis test revealed a significant difference in freezing duration among all groups [χ^2 (7) = 34.508, p<0.001] (Figure 3.5A). However, *post hoc* pairwise analysis with Dunn's test did not reveal significant differences between groups.

Two-way repeated measures ANOVA revealed a significant effect of fear conditioning [F (1, 47) = 37.456, p<0.001], time [F (2.251, 105.816) = 38.350, *p<0.001), and fear conditioning x time [F (2.251, 105.816) = 35.556, p<0.001] on freezing duration analysed as 3-min bins (Figure 3.5B; NFC groups not shown for clarity of presentation). *Post hoc* analysis by Student Newman-Keuls test indicated a significant increase in the duration of freezing in FC GW6471 vs FC Vehicle at 10-12 min ($^{\$}$p<0.05) and at 0-3 min for FC GW9662 vs FC Vehicle ($^{\#}$p<0.05) (Figure 3.5B). There were no significant effects of drug treatment on freezing across time in NFC rats (data not shown). There were no significant effects of treatment [F (3, 47) = 1.750, p>0.05], treatment x conditioning [F (3, 47) = 1.591, p>0.05], time x treatment [F (6.754, 105.816) = 1.538, p>0.05], time x conditioning [F (2.251, 105.816) = 35.556, p>0.05], and time x conditioning x treatment [F (6.754, 105.8160 = 1.372, p>0.05] on freezing duration.

Kruskal Wallis test revealed a significant difference in defecation among all groups [χ^2 (7) = 24.023, p<0.01] (Figure 3.6). However, *post hoc* pairwise analysis with Dunn's test did not reveal significant differences between groups.

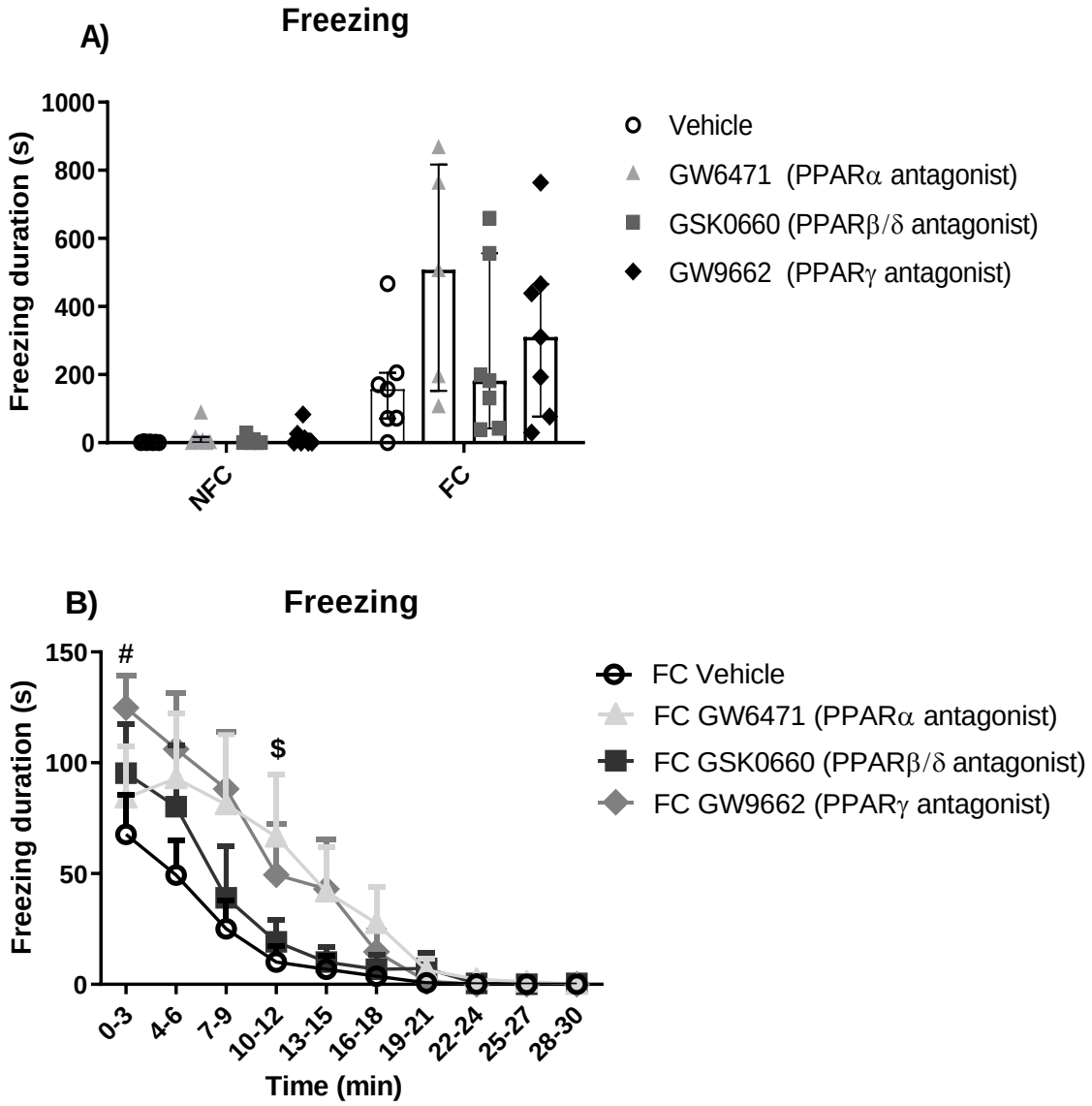


Figure 3.5: Effects of intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on freezing duration over the total trial period (A) and as 3-min time bins (B) in non-fear conditioned (NFC) and fear conditioned (FC) rats. Post hoc analysis indicated a significant increase at 0-3 min for FC GW9662-treated rats (*p<0.05, vs FC Vehicle), and FC GW6471-treated rats at 10-12 min (*p<0.05, vs FC Vehicle). Data are expressed as median with interquartile range and min/max (A) and mean ± S.E.M. (B) (n=7-9 rats per group).

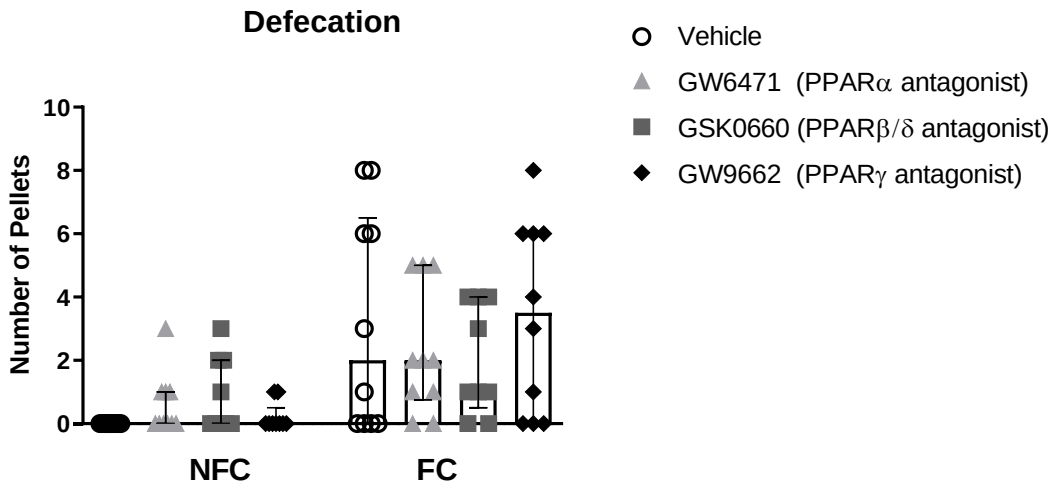


Figure 3.6: Effects of fear conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on defecation. Data are expressed as median with interquartile range and min/max (n= 7-9 rats per group).

3.3.1.4 Intra-BLA administration of GW6471, GSK0660, and GW9662 does not affect general/motor behaviour

Two-way ANOVA showed that there were no significant effects of treatment [F (3, 53) = 0.294, p>0.05], fear conditioning [F (1, 53) = 0.251, p>0.05], and treatment x conditioning [F (3, 53) = 1.425] on walking duration (Figure 3.7A).

Two-way ANOVA showed that there were no significant effects of treatment [F (3, 53) = 0.591, p>0.05], fear conditioning [F (1, 53) = 0.056, p>0.05], treatment x conditioning [F (3, 53) = 0.532, p>0.05] on distance moved (Figure 3.7B).

Kruskal-Wallis test showed no differences among the groups in rearing duration [χ^2 (7) = 5.685, p>0.05] (Figure 3.7C).

Two-way ANOVA showed that there were no significant effects of treatment [F (3, 53) = 0.043, p>0.05], fear conditioning [F (1, 53) = 0.380, p>0.05], and treatment x conditioning [F (3, 53) = 0.268, p>0.05) on grooming duration (Figure 3.7D).

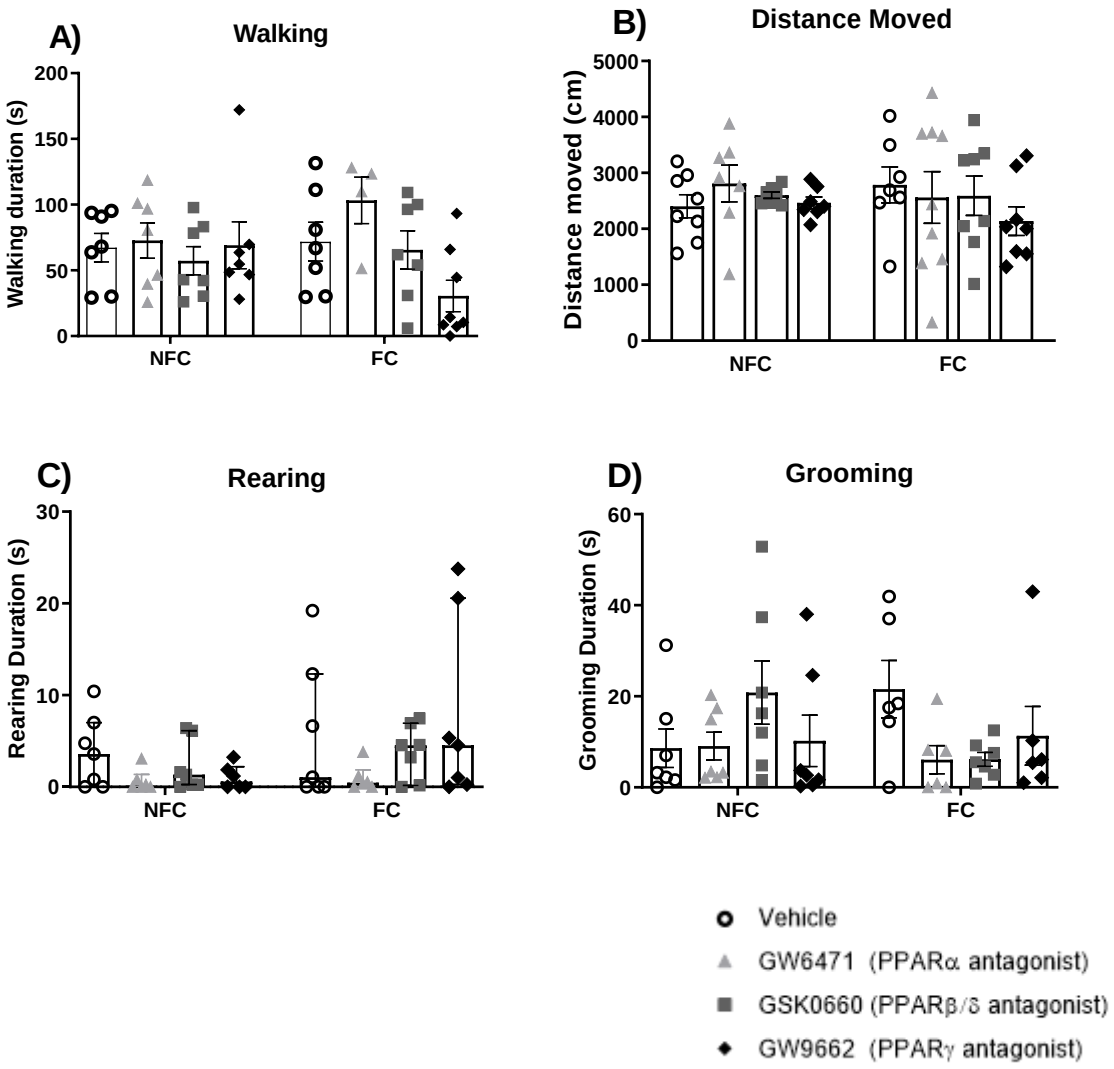


Figure 3.7: Effects of fear-conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on walking duration (A), distance moved (B), grooming duration (C), and rearing duration (D). Data are expressed as mean ± S.E.M. (A, B and D) or median with interquartile range and min/max (C), n=7-9 rats per group.

3.3.1.5 Effect of fear conditioning and PPAR antagonist administration on neurotransmitter levels in the basolateral amygdala (BLA)

Kruskal-Wallis test did not show any significant difference among groups (χ^2 (15) = 20.669, p>0.05) in GABA levels in the BLA (Figure 3.8A). When each side was analysed separately, Kruskal Wallis did not show any significant difference among group in the right [χ^2 (7) = 6.288, p>0.05] or in the left [χ^2 (7) = 5.291, p>0.05] sides in GABA levels in the BLA.

Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (15) = 39.443, $p<0.01$) in glutamate levels in the BLA (Figure 3.8B). However, *post hoc* analysis with Dunn's test did not reach statistical significance. When each side was analysed separately, Kruskal Wallis did not show any significant difference among group in the right [χ^2 (7) = 5.432, $p>0.05$] or in the left [χ^2 (7) = 9.575, $p>0.05$] sides in glutamate levels in the BLA.

Kruskal-Wallis comparisons reveal a significant difference among groups (χ^2 (15) = 84.814, $p<0.001$) in serotonin levels in the BLA (Figure 3.8C). *Post hoc* analysis with Dunn's test indicated that the levels of serotonin were significantly lower in the right BLA of NFC GW6471, FC Vehicle and FC GSK0660 rats compared to their left side counterparts (*$p<0.05$). When each side was analysed separately, Kruskal Wallis showed a significant difference among the groups in the left [χ^2 (7) = 16.134, $p<0.05$] but not in the right [χ^2 (7) = 4.713, $p>0.05$] side in serotonin levels in the BLA. However, *post hoc* pairwise comparisons with Dunn's test did not reach statistical significance.

Kruskal-Wallis test reveal a significant difference among groups (χ^2 (15) = 90.526, $p<0.001$) in dopamine levels in the BLA (Figure 3.8D). *Post hoc* analysis with Dunn's test indicated that the levels of dopamine were significantly lower in the right BLA of NFC Vehicle, FC Vehicle and NFC GSK0660 rats compared to their left counterparts (*$p<0.05$). When each side was analysed separately, Kruskal Wallis did not show any significant difference among groups in the right [χ^2 (7) = 11.912, $p>0.05$] or in the left [χ^2 (7) = 1.796, $p>0.05$] sides in dopamine levels in the BLA.

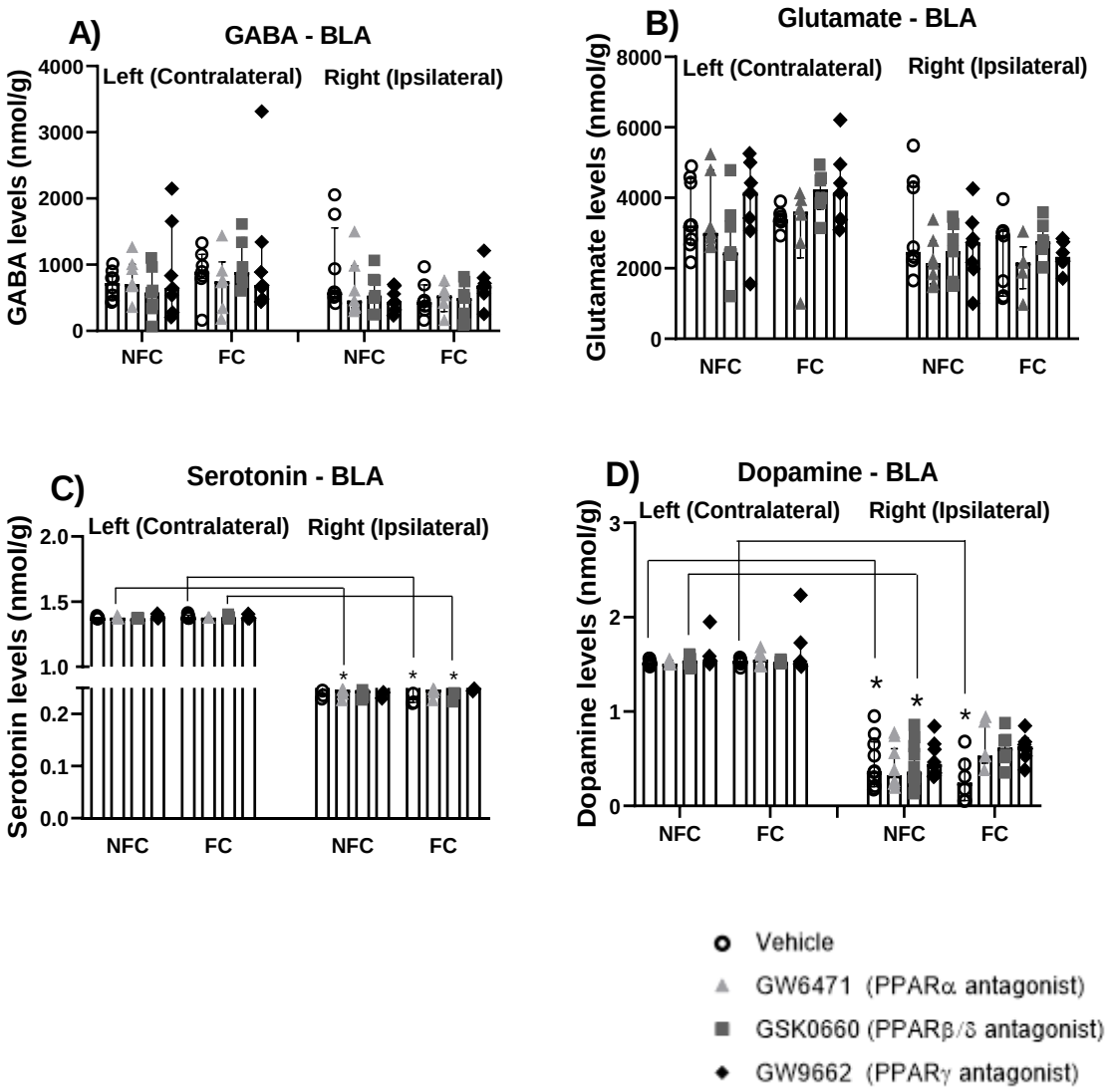


Figure 3.8: Effects of fear-conditioning and intra-BLA administration of PPARα, PPARβ/δ and PPARγ antagonists on the levels of GABA (A), glutamate (B), serotonin (C), and dopamine (D). Post hoc analysis indicated that dopamine levels were significantly lower in the right BLA of NFC Vehicle, FC Vehicle and NFC GSK0660 rats compared to their left counterparts (*p<0.05). Post hoc analysis also indicated that levels of serotonin were lower in the right BLA of NFC GW6471, FC Vehicle and FC GSK0660 rats compared to their left side counterparts (*p<0.05). Data are expressed as median with interquartile range (n=7-9 rats per group).

3.3.1.6 Effect of fear conditioning and PPAR antagonist administration on endocannabinoids and NAE levels in the basolateral amygdala (BLA)

Kruskal-Wallis test did not show any significant difference among groups (χ^2 (15) = 20.097, p>0.05) in 2-AG levels in the BLA (Figure 3.9A). When each side was analysed separately, Kruskal Wallis did not show any significant difference among group in the right [χ^2 (7) = 6.863, p>0.05] or in the left [χ^2 (7) = 7.592, p>0.05] sides in 2-AG levels in the BLA.

Kruskal-Wallis comparisons did not show any significant difference among groups (χ^2 (15) = 22.173, p>0.05) in AEA levels in the BLA (Figure 3.9B). When each side was analysed separately, Kruskal Wallis did not show any significant difference among group in the right [χ^2 (7) = 4.721, p>0.05] or in the left [χ^2 (7) = 6.548, p>0.05] sides in AEA levels in the BLA.

Three-way ANOVA revealed an effect of side [F (1, 84) = 49.888, ^{a}p<0.001] and fear conditioning [F (1, 84) = 4.298, p<0.05] on PEA levels in the BLA (Figure 3.9C). *Post hoc* pairwise analysis with Student Newman-Keuls test did not show any significant statistical differences. There were no significant effects of treatment [F (3, 84) = 0.068, p>0.05], treatment x conditioning [F (3, 84) = 0.669, p>0.05], treatment x side [F (3, 84) = 0.344, p>0.05], conditioning x side [F (1, 84) = 0.074, p>0.05], treatment x conditioning x side [F (3, 84) = 0.656, p>0.05] on PEA levels. When each side was analysed separately, two-way ANOVA did not show any significant effect of treatment, conditioning or their interaction on either the left or right BLA.

Kruskal-Wallis comparisons reveal a significant difference among groups (χ^2 (15) = 31.454, p<0.01) in OEA levels in the BLA (Figure 3.9D). However, *post hoc* analysis with Dunn's test did not reach statistical significance. When each side was analysed separately, Kruskal Wallis test did not show any significant difference among group in the right [χ^2 (7) = 6.672, p>0.05] or in the left [χ^2 (7) = 4.598, p>0.05] sides in OEA levels in the BLA.

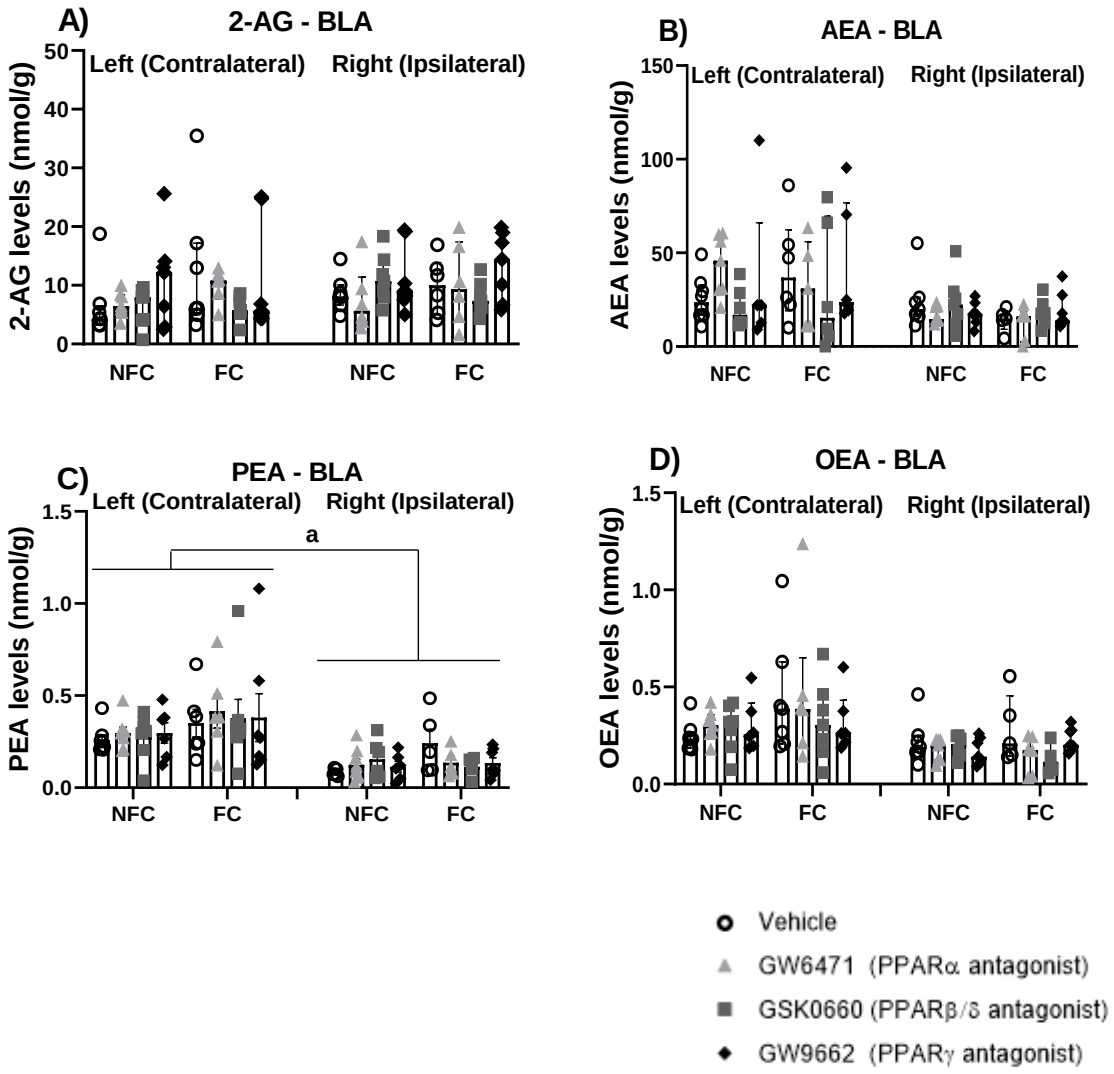


Figure 3.9: Effects of fear-conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on the levels of 2-AG (A), AEA (B), PEA (C), and OEA (D). Two-way ANOVA revealed a significant effect of side on PEA levels ($^{a}p<0.05$). Data are expressed as mean ± S.E.M (C) or median with interquartile range and min/max (A, B and D), (n=7-9 rats per group).

3.3.2 Experiment 2: Effects of intra-BLA administration of PPAR antagonists on conditioned fear in the absence of nociceptive tone in rats

3.3.2.1 Histological verification of microinjection sites

After histological verification, 73% of the rats had both injections correctly placed within the borders of both BLA. Also, 7% had one of the injections in the BLA and the other outside BLA borders. The remaining 20% were placed in the CeA, basomedial amygdala (BMA), or ventral endopiriform nucleus. The data analysed were derived only from rats where intracerebral microinjections were accurately placed in the BLA.

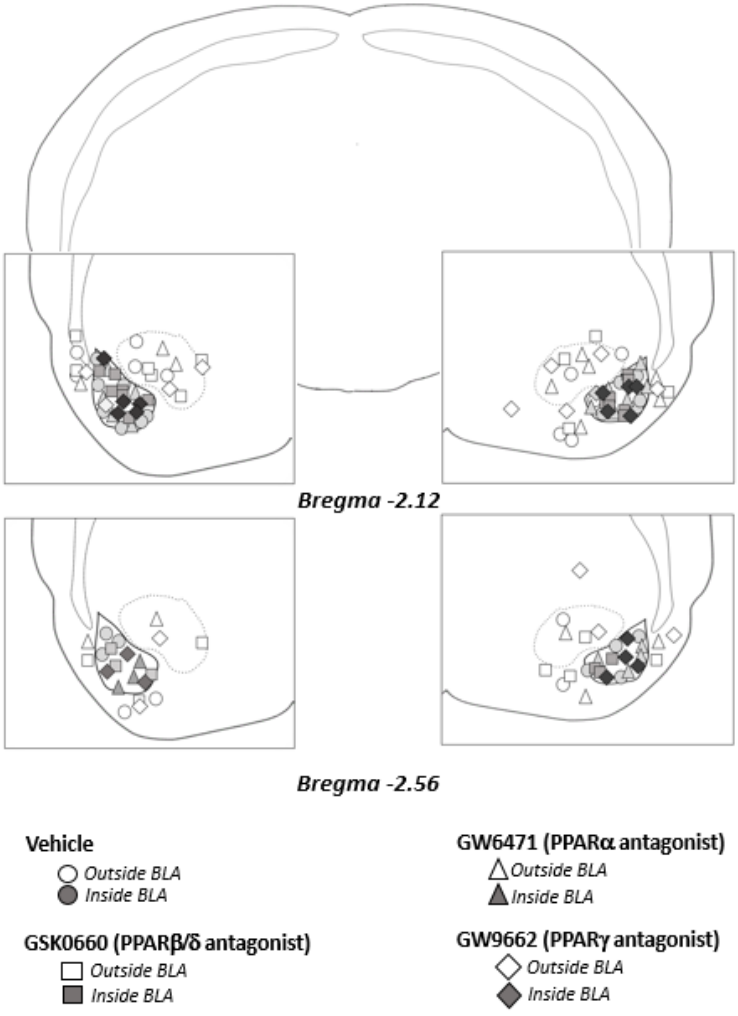


Figure 3.10: Histological verification of injector site location.

3.3.2.2 Intra-BLA administration of PPAR antagonists had no effect on composite pain score in saline-injected rats

Composite pain scores were substantially less in this experiment following intra-plantar saline injection compared with Experiment 1 where rats received intra-plantar formalin injection (Figure 3.11). Kruskal-Wallis test revealed no difference among groups [χ^2 (7) = 4.241, p>0.05] of rats that received an intra-plantar injection of saline into the right hind paw (Figure 3.10). Two-way ANOVA showed that there were no significant effects of fear-conditioning [F (1, 9) = 4.364, p>0.05], treatment [F (3, 27) = 0.5191, p>0.05], or treatment x conditioning [F (3, 26) = 0.4741, p>0.05] on paw diameter (Figure 3.12).

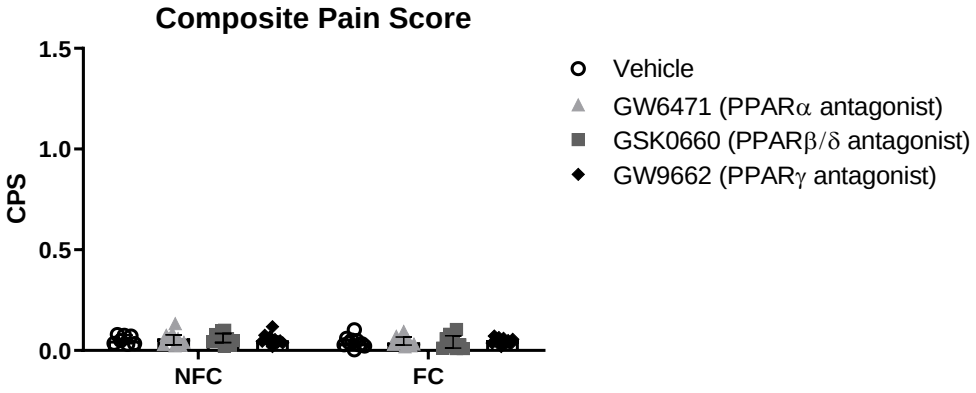


Figure 3.11: Effects of intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on the composite pain score in non-fear conditioned (NFC) and fear conditioned (FC) rats that received intra-plantar injection of saline. Composite pain score was calculated as (pain 1 + 2*[pain 2])/total duration of analysis period (see for further information Material and Methods). Kruskal-Wallis showed no significant difference among groups [χ^2 (7) = 4.241, p>0.05]. Data are expressed as median with interquartile range and min/max (n=8-10 rats per group).

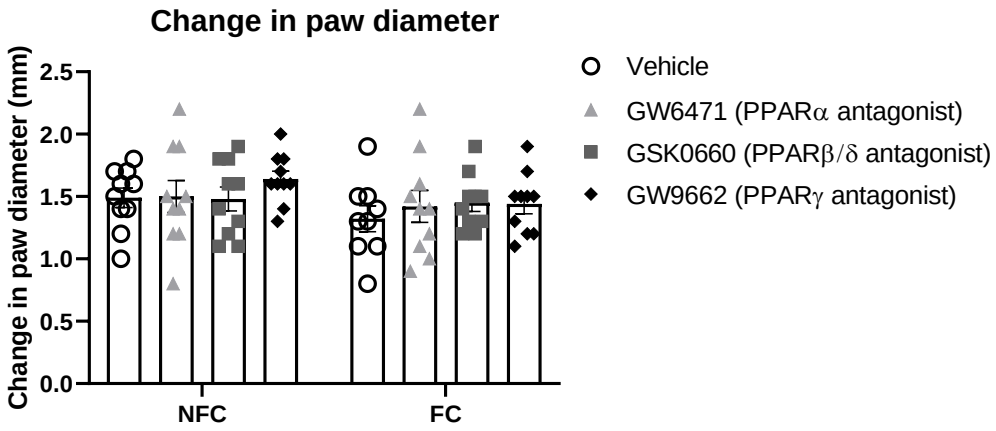


Figure 3.12: Effects of intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on saline-evoked changes in the hind paw diameter in non-fear conditioned (NFC) and fear conditioned (FC) rats. The change was assessed by measuring the paw diameter immediately before, and 60 min after, saline administration. Data are expressed as mean ± S.E.M, n=8-10 rats per group.

3.3.2.3 Intra-BLA administration of PPARs antagonists increases freezing in NFC rats

Kruskal-Wallis revealed a significant difference among groups [χ^2 (7) = 18.037, p=0.012] (Figure 3.13). *Post hoc* pairwise analysis with Dunn's test indicate a significant enhancement in freezing duration in FC vehicle rats compared to their NFC counterparts (NFC Vehicle vs FC Vehicle, *p<0.05). The treatment with GW6471 and GSK0660 in NFC rats also increased freezing duration (NFC Vehicle vs NFC GW6471, **p<0.01; NFC Vehicle vs NFC GSK0660, *p<0.05). The treatment with GW9662 in NFC rats narrowly failed to reached statistical significance (NFC Vehicle vs GW9662, p=0.064). These drugs had no significant effects on FC rats.

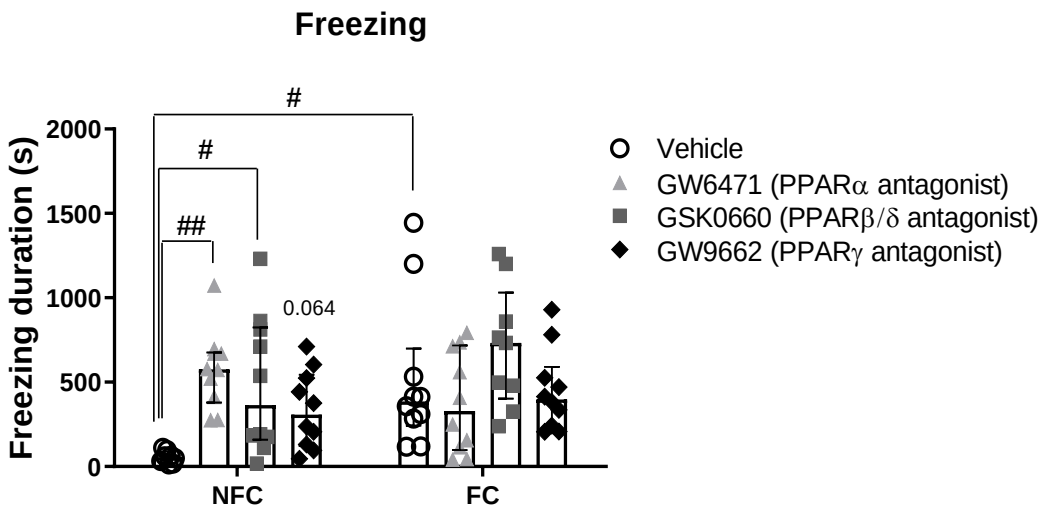


Figure 3.13: Effects of fear conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on total freezing duration. Post hoc indicated an increase in freezing duration in FC vehicle rats ($^{\#}p<0.05$, vs NFC Vehicle). The treatment with GW6471 and GSK0660 in NFC rats also increased freezing duration ($^{\#\#}p<0.01$ vs NFC Vehicle; $^{\#}p<0.05$ vs NFC Vehicle). Treatment with GW9662 almost reached statistical significance (p=0.064, vs NFC Vehicle). Data are expressed as median with interquartile range and min/max (n= 7-9 rats per group).

Kruskal-Wallis analysis of the defecation data revealed a significant difference among groups [χ^2 (7) = 23.49, p<0.01] (Figure 3.14). *Post hoc* pairwise analysis with Dunn's test did not show any significant difference between groups.

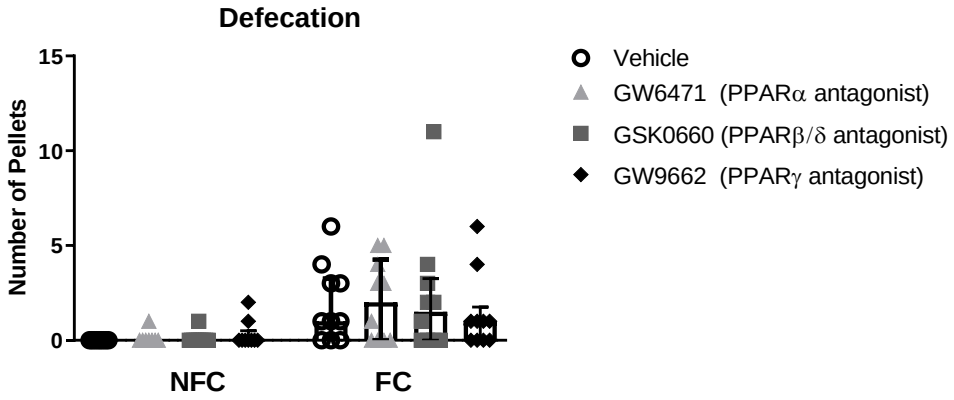


Figure 3.14: Effects of fear conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on defecation. Data are expressed as median with interquartile range (n=7-9 rats per group).

3.3.2.4 Intra-BLA administration of PPAR antagonists does not affect general/motor behaviour

Two-way ANOVA showed that there were no significant effects of treatment [F (3, 58) = 0.332, p>0.05], fear conditioning [F (1, 58) = 0.133, p>0.05], and treatment x conditioning [F (3, 58) = 0.244, p>0.05] on walking duration (Figure 3.15A).

Two-way ANOVA showed that there were no significant effects of treatment [F (3, 58) = 0.716, p>0.05], fear conditioning [F (1, 58) = 0.055, p>0.05], and treatment x conditioning [F (3, 58) = 1.199, p>0.05] on distance moved (Figure 3.15B).

Two-way ANOVA showed an effect of treatment [F (3, 50) = 3.686, p<0.05] on rearing duration (Figure 3.15C). However, *post hoc* analysis with Student Newman-Keuls did not reveal significant statistical differences between groups. There were no significant effects of fear conditioning [F (1, 50) = 0.261, p>0.05] and treatment x conditioning [F (3, 50) = 0.256, p>0.05] on rearing duration.

Two-way ANOVA showed that there were no significant effects of treatment [F (3, 50) = 0.628, p>0.05], fear conditioning [F (1, 50) = 0.053, p>0.05], and treatment x conditioning [F (3, 50) = 0.248, p>0.05) on grooming duration (Figure 3.15D).

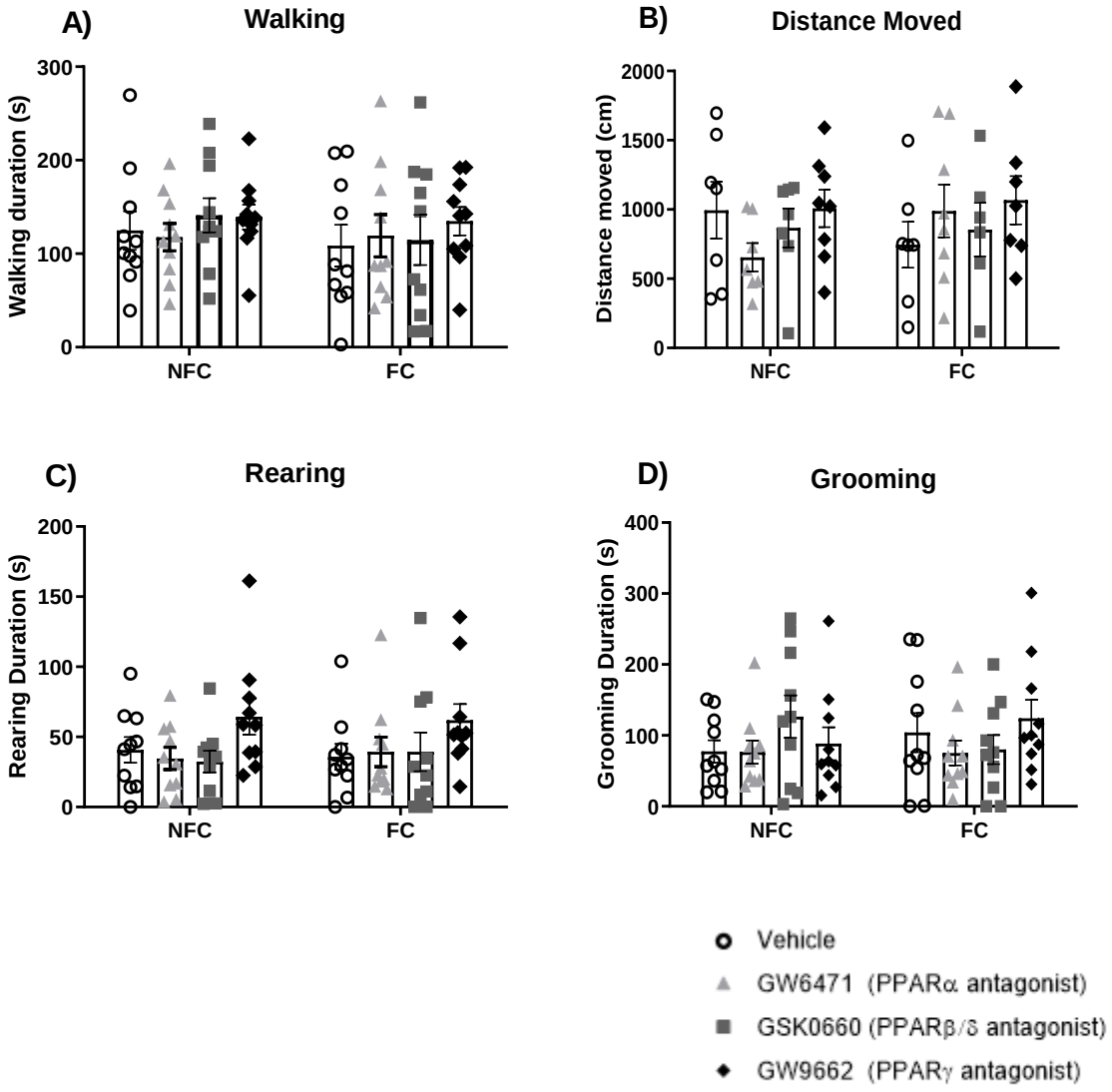


Figure 3.15: Effects of fear-conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on walking duration (A), distance moved (B), grooming duration (C), and rearing duration (D). Data are expressed as mean ± S.E.M (n=7-9 rats per group).

3.3.2.5 Effect of fear conditioning and PPAR antagonist administration on neurotransmitter levels in the basolateral amygdala (BLA)

Kruskal-Wallis test revealed a significant difference among groups (χ^2 (15) = 25.622, $p<0.05$) in GABA levels (Figure 3.16A) in the BLA. However, *post hoc* analysis with Dunn's test did not show any significant between-group differences in GABA levels. When each side was analysed separately, Kruskal Wallis did reveal a significant difference among

groups in the right [χ^2 (7) =14.483, $p<0.05$] but not in the left [χ^2 (7) = 3.012, $p>0.05$] side. However, *post hoc* analysis with Dunn's test did not show any significant between-group differences in GABA levels.

Kruskal-Wallis test did not show any significant difference among groups (χ^2 (15) = 15.856, $p>0.05$) in glutamate levels (Figure 3.16B) in the BLA. When each side was analysed separately, Kruskal Wallis did not reveal a significant difference among groups in the right [χ^2 (7) = 6.458, $p>0.05$] or in the left [χ^2 (7) = 3.802, $p>0.05$] side.

Kruskal-Wallis test did not show any significant difference among groups (χ^2 (15) = 22.532, $p>0.05$) in serotonin levels (Figure 3.16C) in the BLA. When each side was analysed separately, Kruskal Wallis did not reveal a significant difference among group in the right [χ^2 (7) = 12.250, $p>0.05$] or in the left [χ^2 (7) = 2.039, $p>0.05$] side.

Kruskal-Wallis test revealed a significant difference among groups (χ^2 (15) = 58.963, $p<0.001$) in dopamine levels (Figure 3.16D) in the BLA. *Post hoc* analysis with Dunn's test indicated that NFC GW9662-treated rats have higher levels of dopamine levels in the right side compared to the left side ($^{*}p<0.05$). When each side was analysed separately, Kruskal Wallis did not reveal a significant difference among group in the right [χ^2 (7) = 11.644, $p=0.053$] and in the left [χ^2 (7) = 8.987, $p>0.05$] side. Because the right side almost reached statistical difference, an analysis considering the different fear conditioning groups was carried out. When we further analyse the fear conditioning groups, Kruskal Wallis test revealed a significant difference among groups in the NFC rats in the right [χ^2 (3) = 8.324, $p<0.05$] but not in the left [χ^2 (3) = 5.168, $p>0.05$] side. *Post hoc* analysis with Dunn's test indicated that NFC rats treated with GW6471 have increased dopamine levels compared to NFC Vehicle-treated ones ($^{\#}p<0.05$). The test also indicated a strong trend for increased levels of dopamine in NFC GW9662-treated rats compared to NFC vehicle-treated ($p=0.0584$). Kruskal Wallis test did not reveal any significant differences among groups in FC rats neither in the right [χ^2 (3) = 1.937, $p>0.05$] nor in the left [χ^2 (3) = 3.028, $p>0.05$] side.

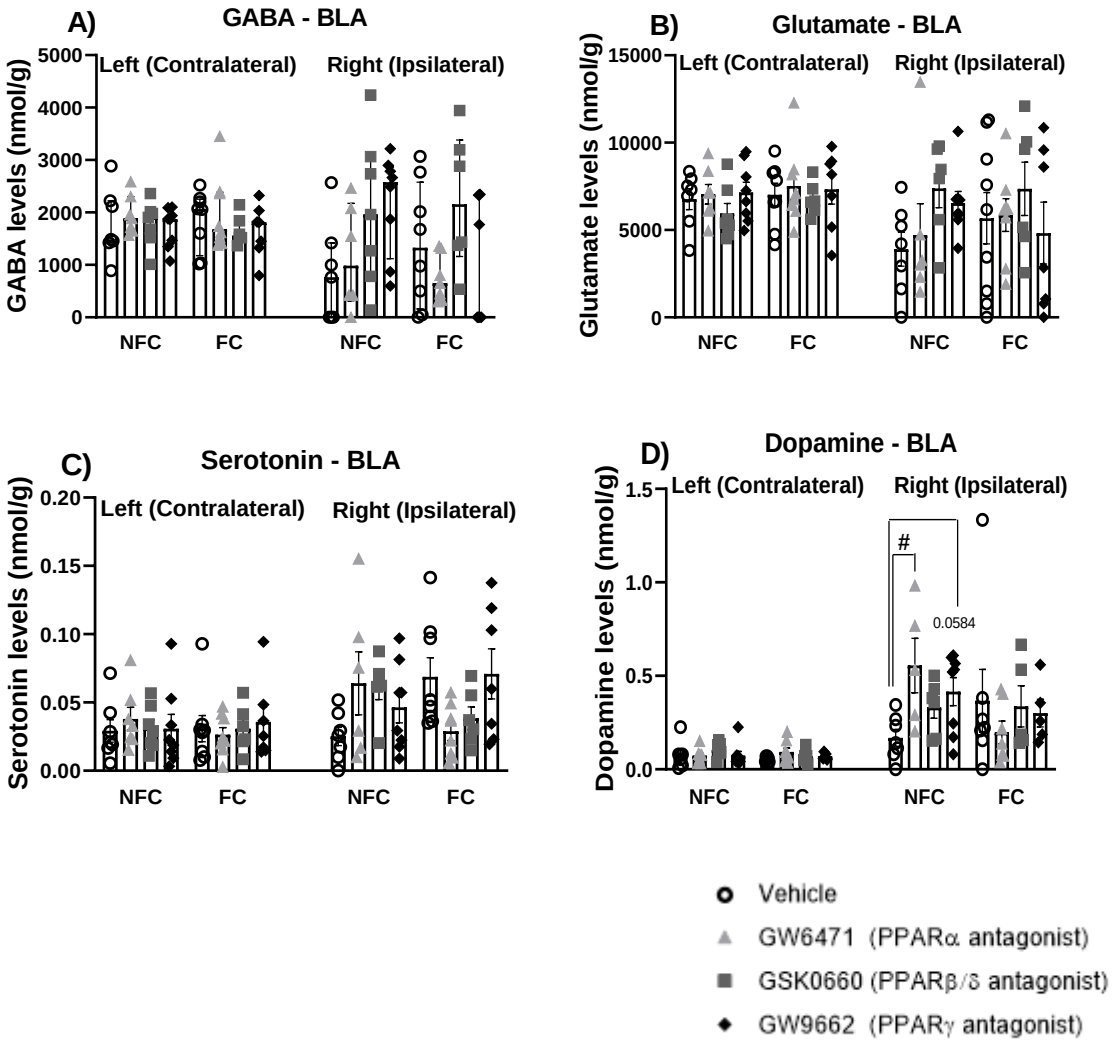


Figure 3.16: Effects of fear-conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on the tissue levels of GABA (A), glutamate (B), serotonin (C), and dopamine (D) in the BLA. Post hoc analysis indicated that NFC rats treated with GW6471 have increased dopamine levels compared to NFC Vehicle-treated ones ($^{\#}p<0.05$). The test also indicated a strong trend for increased levels of dopamine in NFC GW9662-treated rats compared to NFC vehicle-treated ($p=0.0584$). Data are expressed as median with interquartile range and min/max (n=7-9 rats per group).

3.3.2.6 Effect of fear conditioning and PPAR antagonist administration on endocannabinoid and NAE levels in the basolateral amygdala (BLA)

Kruskal-Wallis comparisons did not show any significant difference among groups (χ^2 (15) = 18.374, $p>0.05$) in 2-AG levels (Figure 3.17A) in the BLA. When each side was analysed

separately, Kruskal Wallis did not reveal a significant difference among group in the right [χ^2 (7) = 9.526, p>0.05] and in the left [χ^2 (7) = 4.186, p>0.05] side.

Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (15) = 47.410, p<0.05) in AEA levels (Figure 3.17B) in the BLA. However, *post hoc* analysis with Dunn's test did not show any significant changes in AEA levels. When each side was analysed separately, Kruskal Wallis revealed a significant difference among group in the right [χ^2 (7) =14.798, p<0.05] but not in the left [χ^2 (7) = 6.537, p>0.05] side. However, *post hoc* analysis with Dunn's test did not show any significant changes in AEA levels in the Right BLA.

Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (15) = 32.124, p<0.05) in PEA levels (Figure 3.17C) in the BLA. However, *post hoc* analysis with Dunn's test did not show any significant changes in PEA levels. When each side was analysed separately, two-way ANOVA revealed a significant effect of treatment [F (3, 44) = 3.034, p<0.05], fear conditioning [F (1, 44) = 7.163, p<0.05) and the interaction of treatment x fear conditioning [F (3, 44) = 3.606, p<0.05] on PEA levels in the right BLA. *Post hoc* analysis with Dunn's test indicated that FC GW6471-treated rats have decreased levels of PEA compared to FC Vehicle treated rats in the right BLA ($^{\$}$p<0.05). Two-way ANOVA did not reveal any significant effect of treatment [F (3, 48) = 0.624, p>0.05], fear conditioning [F (1, 48) = 0.590, p<0.05] and the interaction of treatment x fear conditioning [F (3, 48) = 0.830, p<0.05] on PEA levels in the left BLA.

Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (15) = 32.456, p<0.05) in OEA levels (Figure 3.17D) in the BLA. However, *post hoc* analysis with Dunn's test did not show any significant changes in OEA levels. When each side was analysed separately, Kruskal Wallis test revealed a significant difference among groups in OEA levels in the right (χ^2 (7) = 21.988, p<0.01) but not in the left (χ^2 (7) = 6.350, p>0.05) BLA. *Post hoc* analysis with Dunn's did not reveal any significant differences between groups in OEA levels in the right BLA.

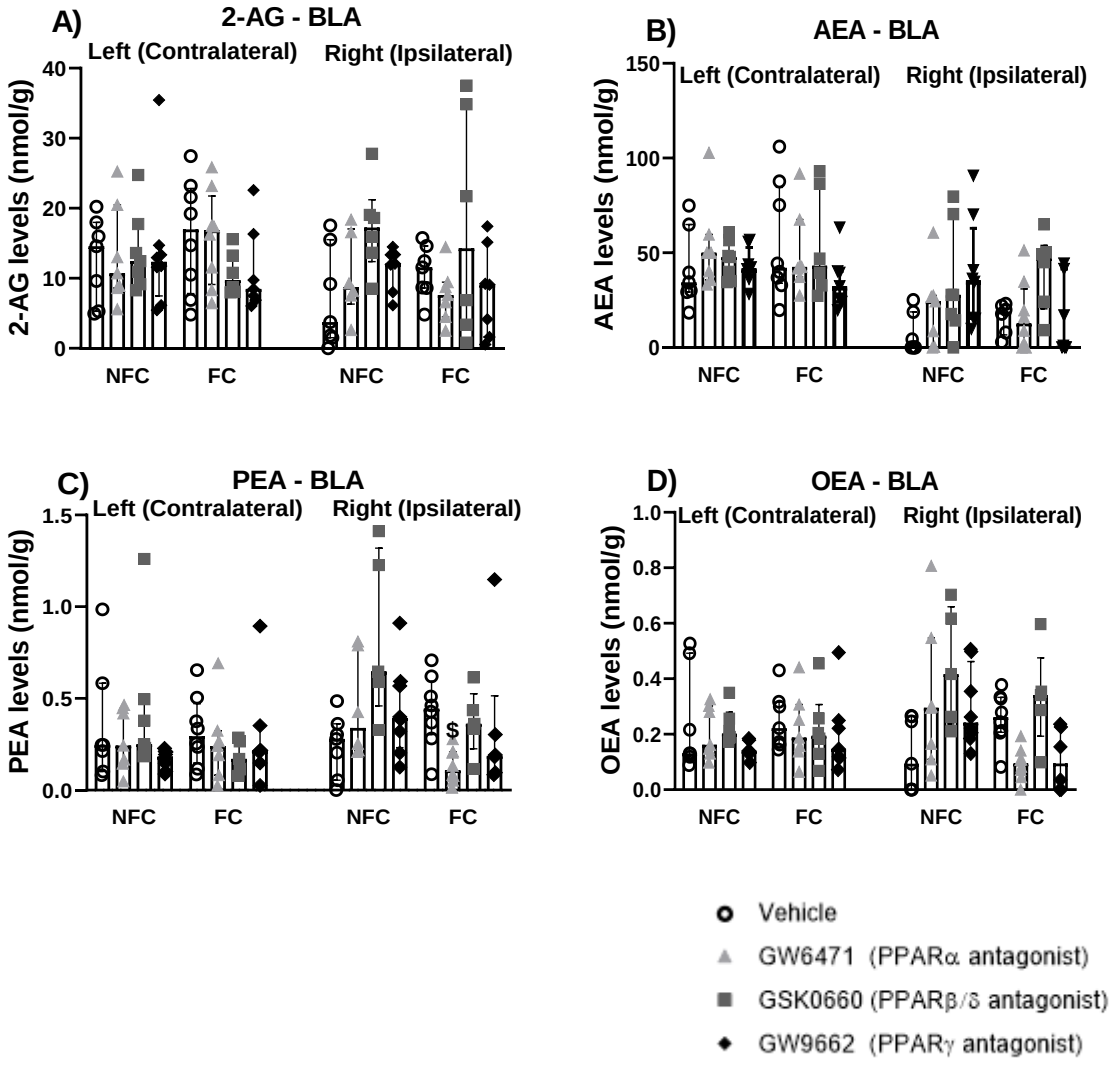


Figure 3.17: Effects of fear-conditioning and intra-BLA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on the levels of 2-AG (A), AEA (B), PEA (C), and OEA (D). Post hoc analysis with Dunn's test indicated that FC GW6471-treated rats have decreased levels of PEA compared to FC Vehicle treated rats in the right BLA ($^{\$}p<0.05$). Data are expressed as median with interquartile range (n=6-9 rats per group).

3.3.3 Expression of PPARs in the BLA

3.3.1.1 Western Blotting

PPARα, PPARβ/δ and PPARγ expression was confirmed in the right and left BLA of naïve male SD rats (Figure 3.18). The bands for PPARα (55kDa) and PPARβ/δ (52kDa) were obtained with the use of a monoclonal antibody. As mentioned in the section 3.2.9.1, the double bands for PPARγ are a consequence of the expression of two subtypes of PPARγ:

PPARγ_1 and PPARγ_2. The 42kDa band corresponds to β-actin, used as an endogenous control.

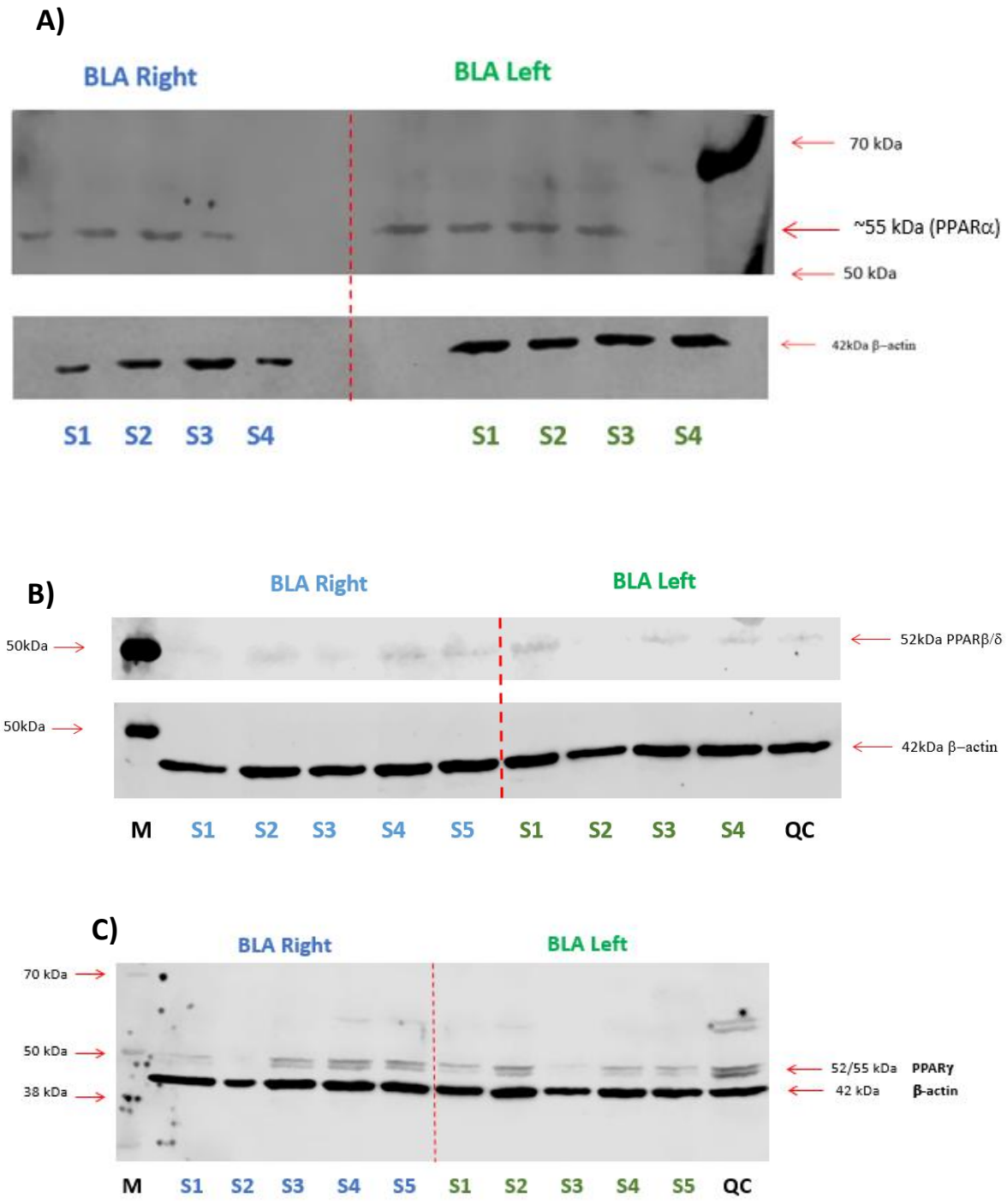


Figure 3.18: Expression of PPARα, PPARβ/δ, and PPARγ in the right and left BLA (n=4-5 per side). The expression of PPARα is seen at 55kDa, PPARβ/δ at 52kDa, and PPARγ at 52/55kDa. β-actin was used as endogenous control. M=marker/ladder; QC=quality control.

3.3.1.1 RT-qPCR

The available antibodies developed to bind to PPARβ/δ in western blotting protocols did not give results that were entirely satisfactory, as evidenced by the faint bands above (Figure 3.18C). Therefore, we opted to demonstrate the presence of PPARβ/δ in the BLA using RT-qPCR. The presence of mRNA encoding PPARβ/δ was confirmed in the right and left BLA of naïve male SD rats. The Ct values found for the BLA punches were 30.046±0.11 in the BLA Right and 29.741±0.02 in the BLA Left. Data are expressed as means ± S.Dev (Figure 3.19)

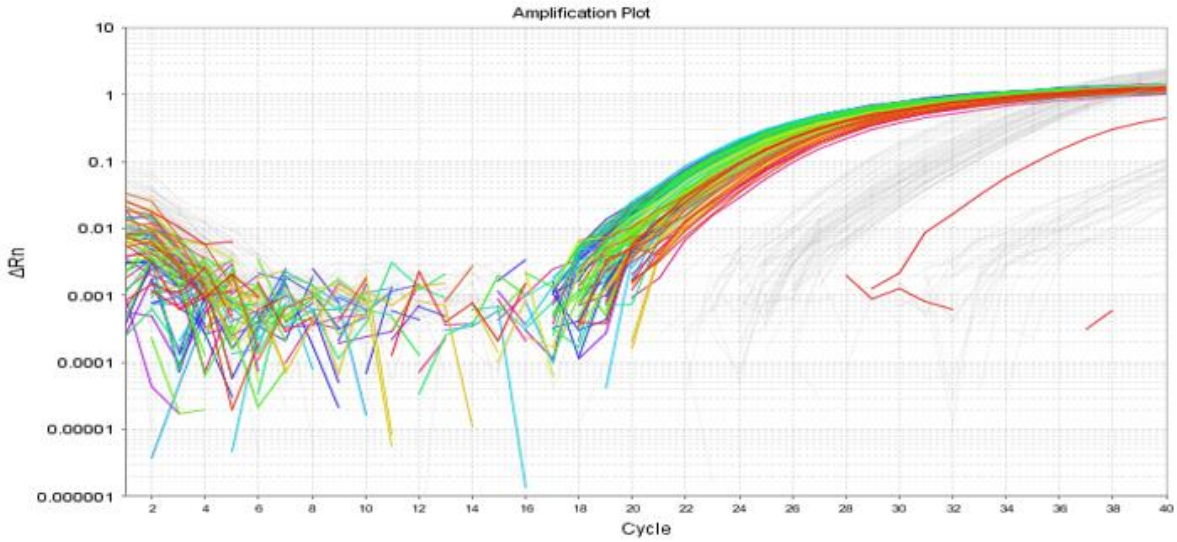


Figure 3.19: Amplification plots for PPARβ/δ gene expression in the right and left BLA.

3.3.4 Effects of intra-plantar administration of formalin on levels of neurotransmitters, endocannabinoids and NAEs in NFC and FC rats

Samples from the NFC and FC vehicle-treated groups from both experiments were re-run on LC-MS/MS and re-analysed together in order to compare possible effects of the presence of a nociceptive inflammatory tone (i.e. formalin) *versus* its absence (i.e. saline) on the levels of neurotransmitters, endocannabinoids and NAEs.

3.3.4.1 Effects of intra-plantar (i.pl.) administration of formalin on levels of neurotransmitters in NFC and FC rats

Three-way ANOVA revealed a significant main effect of side [$F(1, 52) = 10.730$, $^{a}p=0.002$] on GABA levels in the BLA (Figure 3.20A). *Post hoc* pairwise analysis with Student Newman-Keuls did not show any significant statistical differences. There were no significant effects of fear conditioning [$F(1, 52) = 0.003$, $p>0.05$], treatment [$F(1, 52) = 2.446$, $p>0.05$], treatment x conditioning [$F(1, 52) = 2.030$, $p>0.05$], treatment x side [$F(1, 52) = 0.022$, $p>0.05$], conditioning x side [$F(1, 52) = 0.556$, $p>0.05$], treatment x conditioning x side [$F(1, 52) = 3.365$, $p>0.05$] on GABA levels. When the right and left sides were analysed separately, two-way ANOVA did not show any significant effect of treatment, conditioning or their interaction on GABA levels in either left or right BLA.

Three-way ANOVA revealed an effect of side [$F(1, 52) = 5.630$, $^{a}p=0.021$] on glutamate levels in the BLA (Figure 3.20B). *Post hoc* pairwise analysis with Student-Newman-Keuls did not show any significant statistical differences. There were no significant effects of fear conditioning [$F(1, 52) = 0.103$, $p>0.05$], treatment [$F(1, 52) = 0.865$, $p>0.05$], treatment x conditioning [$F(1, 52) = 1.429$, $p>0.05$], treatment x side [$F(1, 52) = 0.637$, $p>0.05$], conditioning x side [$F(1, 52) = 0.007$, $p>0.05$], treatment x conditioning x side [$F(1, 52) = 1.133$, $p>0.05$] on glutamate levels. When the contra and left sides were analysed separately, two-way ANOVA did not show any significant effect of treatment, conditioning or their interaction on glutamate in either left or right BLA.

Three-way ANOVA revealed an effect of side [$F(1, 51) = 12.192$, $^{a}p=0.001$] and fear conditioning [$F(1, 51) = 5.238$, $p=0.026$] on serotonin levels in the BLA (Figure 3.20C). *Post hoc* pairwise analysis with Student Newman-Keuls indicated that saline-treated FC rats have increased levels of serotonin compared to their NFC counterparts (NFC Saline-treated vs FC Saline-treated, $^{\#}p<0.05$) on the right side. There were no significant effects of treatment [$F(1, 51) = 0.029$, $p>0.05$], treatment x conditioning [$F(1, 51) = 1.564$, $p>0.05$], treatment x side [$F(1, 51) = 1.644$, $p>0.05$], conditioning x side [$F(1, 51) = 2.044$, $p>0.05$], treatment x conditioning x side [$F(1, 51) = 3.796$, $p>0.05$] on serotonin levels. When right and left sides were analysed separately, two-way ANOVA revealed significant effect of fear conditioning [$F(1, 24) = 4.464$, $^{£}p<0.05$] on serotonin levels in the right BLA. However, p*ost hoc* pairwise analysis with Student Newman-Keuls did not show significant statistical differences. Two-way ANOVA showed that there were no significant effects of treatment, conditioning and their interaction on serotonin levels in the left BLA.

Three-way ANOVA revealed an effect of side [F (1, 47) = 53.882, $^{a}p<0.001$] and treatment [F (1, 47) = 14.541, $p<0.001$] on dopamine levels in the BLA (Figure 3.20D). *Post hoc* pairwise analysis with Student Newman-Keuls confirmed the side differences (*$p<0.05$, compared to their left counterparts) and indicated that NFC rats which received an intraplantar injection of formalin have increased levels of dopamine on the right BLA (NFC Saline-treated vs NFC-Formalin-treated, $^{\#}p<0.05$). There were no significant effects of fear conditioning [F (1, 47) = 0.002, $p>0.05$], treatment x conditioning [F (1, 47) = 0.055, $p>0.05$], treatment x side [F (1, 47) = 2.115, $p>0.05$], conditioning x side [F (1, 47) = 0.477, $p>0.05$], treatment x conditioning x side [F (1, 47) = 1.358, $p>0.05$] on dopamine levels. When the right and left sides were analysed separately, two-way ANOVA did not show any significant effect of treatment, conditioning or their interaction on dopamine levels in either left or right BLA.

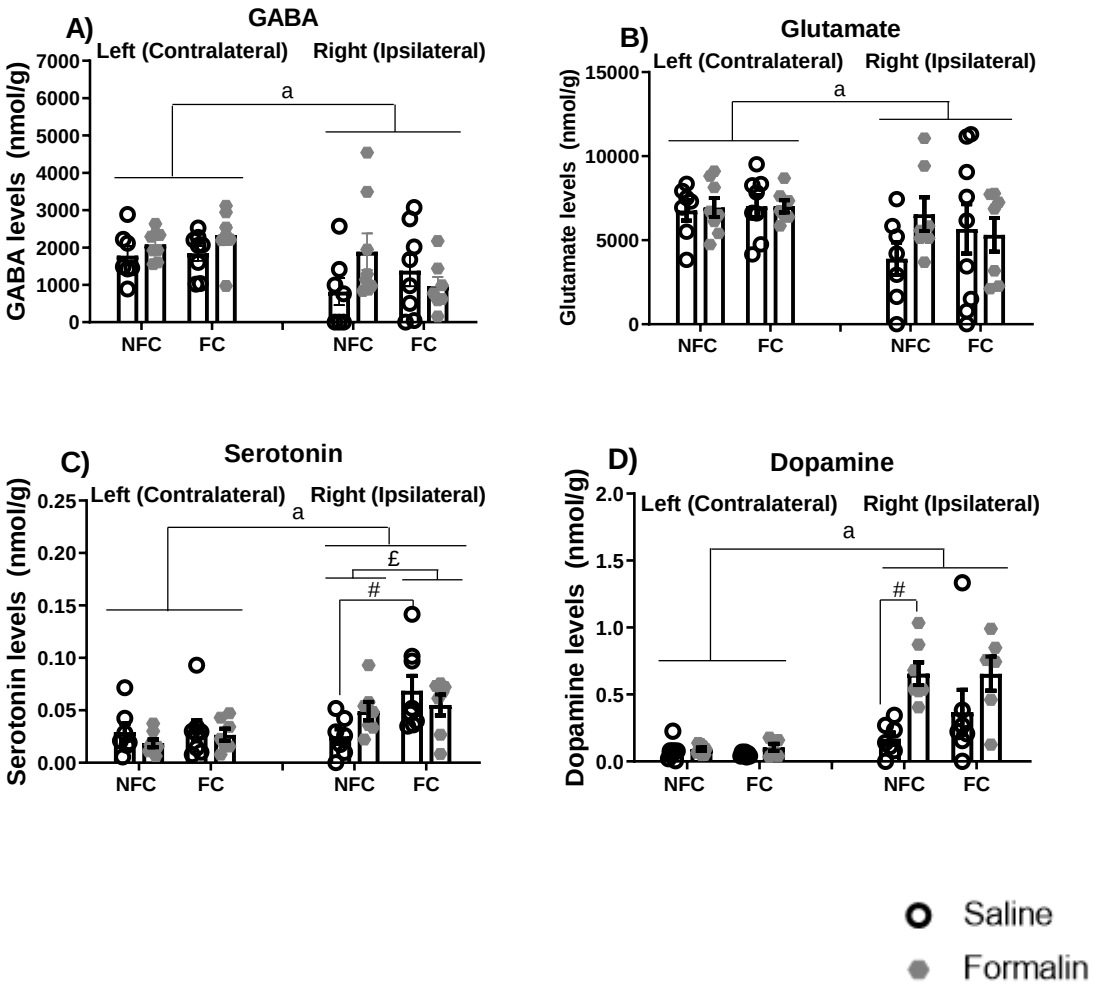


Figure 3.20: Effects of fear-conditioning and intraplantar injection of formalin on the levels of GABA (A), glutamate (B), serotonin (C), and dopamine (D). Two-way ANOVA revealed a significant effect of side on all neurotransmitters ($^{a}p<0.05$). Post hoc pairwise analysis with Student-Newman-Keuls showed a significant difference in serotonin levels between NFC Vehicle and FC Vehicle of i.pl. saline-treated rats ($^{\#}p<0.05$), and dopamine levels between NFC Vehicle i.pl. saline-treated and NFC Vehicle i.pl. formalin-treated rats ($^{\#}p<0.05$). The test also confirmed side differences (*$p<0.05$, compared to their left counterparts) in dopamine levels. Data are expressed as mean ± S.E.M (n=7-9 rats per group).

3.3.4.2 Effects of intra-plantar administration of formalin on levels of endocannabinoids and NAEs in NFC and FC rats

Three-way ANOVA revealed an effect of treatment x side [F (1, 48) = 4.200, p=0.046] on 2-AG levels in the BLA (Fig. 3.21A). *Post hoc* pairwise analysis with Student Newman-Keuls did not show any significant statistical differences. There were no significant effects of fear conditioning [F (1, 48) = 3.971, p>0.05], treatment [F (1, 48) = 3.609, p>0.05], side [F (1, 48) = 2.304, p>0.05], treatment x conditioning [F (1, 48) = 0.646, p>0.05], conditioning x side [F (1, 48) = 0.133, p>0.05], treatment x conditioning x side [F (1, 48) = 0.639, p>0.05] on 2-AG levels. When right and left sides were analysed separately, two-way ANOVA revealed significant effect of treatment [F (1, 26) = 5.401, p=0.028] on 2-AG levels in the left BLA. However, p*ost hoc* pairwise analysis with Student Newman-Keuls did not show significant statistical differences. Two-way ANOVA showed that there were no significant effects of treatment, conditioning and their interaction on 2-AG levels in the right BLA.

Kruskal-Wallis comparisons revealed a significant difference among groups (χ^2 (7) = 35.131, p<0.05) in AEA levels (Figure 3.21B). *Post hoc* analysis with Dunn's test showed lower levels of AEA of NFC Saline group in the right side compared to the left (*p<0.05). When each side was analysed separately, Kruskal Wallis did not reveal any significant differences among group in the right [χ^2 (3) =6.485, p>0.05] or in the left [χ^2 (3) = 2.456, p>0.05] side.

Three-way ANOVA revealed an effect of side [F (1, 49) = 4.191, ^{a}p=0.046] on PEA levels in the BLA (Fig. 3.21C). *Post hoc* pairwise analysis with Student Newman-Keuls did not show significant statistical differences. There were no significant effects of fear conditioning [F (1, 49) = 3.237, p>0.05], treatment [F (1, 49) = 3.912, p>0.05], treatment x conditioning [F (1, 49) = 0.035, p>0.05], treatment x side [F (1, 49) = 3.758, p>0.05], conditioning x side [F (1, 49) = 0.856, p>0.05], treatment x conditioning x side [F (1, 49) = 1.275, p>0.05] on PEA levels. When right and left sides were analysed separately, two-way ANOVA revealed significant effect of treatment [F (1, 23) = 8.216, p=0.009] on PEA levels in the right BLA. *Post hoc* pairwise analysis with Student-Newman-Keuls indicated that FC rats that received formalin injection had lower levels of PEA in the right side compared to their saline-treated counterparts (FC Formalin-treated vs FC Saline-treated, $^{\$}$p<0.05). Two-way ANOVA showed that there were no significant effects of treatment, conditioning or their interaction on PEA levels in the left BLA.

Three-way ANOVA revealed an effect of side [F (1, 48) = 9.699, [a]p=0.003] on OEA levels in the BLA (Fig. 3.21D). *Post hoc* pairwise analysis with Student Newman-Keuls did not show significant statistical differences. There were no significant effects of fear conditioning [F (1, 48) = 3.013, p>0.05], treatment [F (1, 48) = 0.346, p>0.05], treatment x conditioning [F (1, 48) = 0.087, p>0.05], treatment x side [F (1, 48) = 2.259, p>0.05], conditioning x side [F (1, 48) = 0.308, p>0.05], treatment x conditioning x side [F (1, 48) = 1.667, p>0.05] on OEA levels. When the right and left sides were analysed separately, two-way ANOVA showed that there were no significant effects of treatment, conditioning or their interaction on OEA levels in either the left or right BLA.

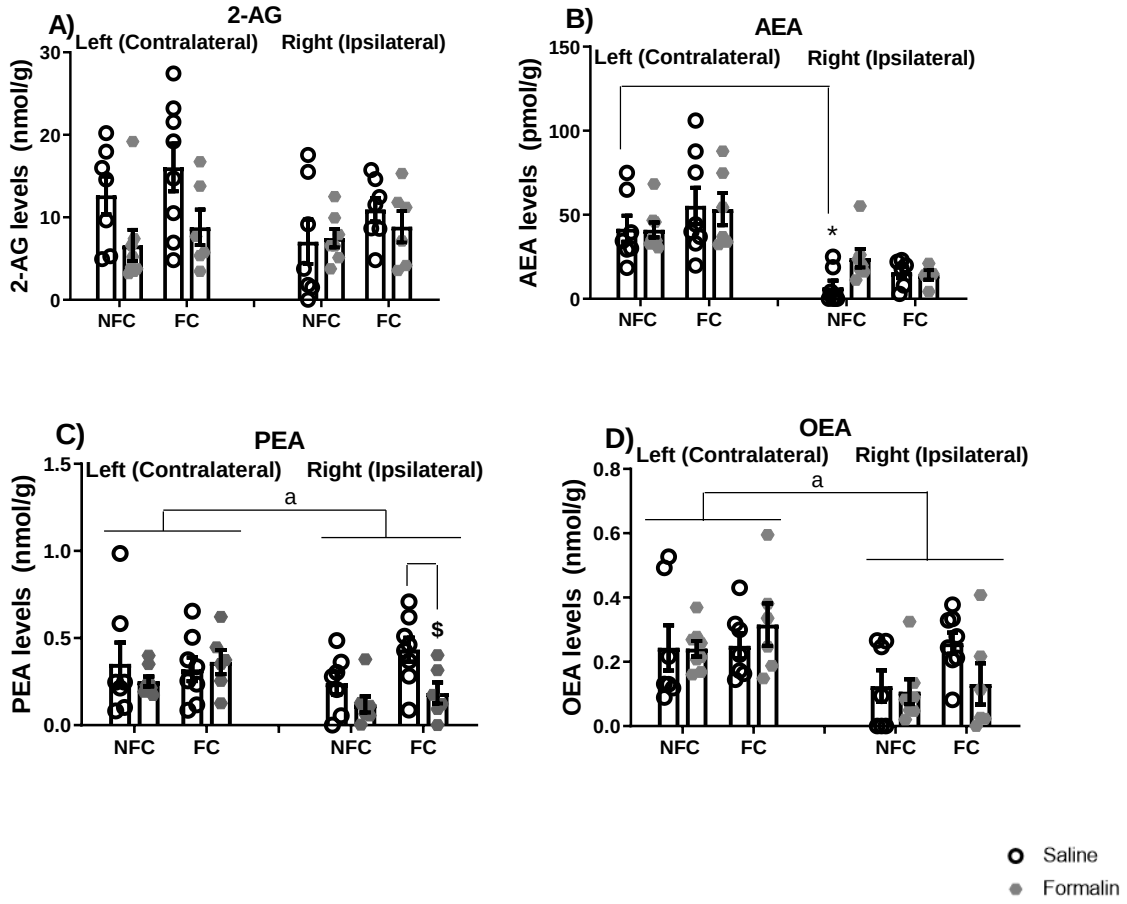


Figure 3.21: Effects of fear-conditioning and intraplantar injection of formalin on the levels of 2-AG (A), AEA (B), PEA (C), and OEA (D). Two-way ANOVA revealed a significant effect of side on PEA and OEA ([a]p<0.05). Post hoc pairwise analysis with Student-Newman-Keuls indicated that FC rats that received formalin injection had lower levels of PEA in the right side compared to their saline-treated counterparts (FC Formalin-treated vs FC Saline-treated, [$]p<0.05). Data are expressed as mean ± S.E.M (A, C, D) or median with interquartile range (B); n=6-9 rats per group.

3.4 Discussion

The two experiments described in this chapter investigated a possible role of PPARs expressed in the BLA in the mediation or modulation of inflammatory pain, FCA, and conditioned fear, the latter in the presence and absence of nociceptive tone. The expression of the three isoforms in the BLA was confirmed by western blotting (and RT-qPCR in the case of PPARβ/δ). Administration of GW6471, a PPARα antagonist, directly into the BLA prolonged freezing duration in FC rats in the presence of formalin-evoked nociceptive tone and increased freezing duration in NFC rats in the absence of nociceptive tone. The administration of a PPARγ antagonist, GW9662, into the BLA enhanced freezing expression in the first part of the trial in the presence, but not in the absence, of nociceptive tone. Thus, endogenous PPAR signalling through PPARα expressed in the BLA may act to attenuate or extinguish conditioned fear behaviour. Likewise, PPAR signalling through PPARγ expressed in the BLA seems to be involved in the recall of fear-related memories, with its blockade resulting in potentiation of fear conditioned behaviour in the first part of the trial. Importantly, these effects are only seen in the presence of formalin-evoked nociceptive tone; they were not observed in rats that received intra-plantar saline injection. The effects of PPARα and PPARγ blockade on freezing expression were associated with increased levels of dopamine in the right BLA. In the absence of nociceptive tone, the administration of the three antagonists increased freezing duration in NFC rats. These results suggest a modulatory role for PPARs in innate anxiety, but not in conditioned fear, in the absence of nociceptive tone. The intra-BLA injection of PPAR antagonists did not alter nociceptive behaviour or locomotor activity in either NFC or FC rats, irrespective of the nociceptive status. These results suggest that PPAR signalling in the BLA does not modulate pain or FCA. Taken together, these results demonstrate a differential effect of the PPAR signalling system on fear and/or anxiety in the presence versus absence of acute inflammatory pain.

Extinction is defined as a learned inhibition of retrieval of previously acquired memories. As discussed in chapter 2, many studies have demonstrated that PPAR signalling plays a role in mnemonic formation (Campolongo et al., 2009a; Mazzola et al., 2009b; Ratano et al., 2017; Chikahisa et al., 2019). However, the role of PPARs expressed in the BLA in memory and learning formation have not been investigated yet. We propose that the blockade of PPARα expressed in the BLA delayed short-term, within-trial extinction of fear memory in the presence of nociceptive tone. The blockade of PPARγ in the same region

potentiated the initial freezing expression, but did not affect its extinction, in the presence of a nociceptive tone. These effects are related to increased levels of dopamine in the right BLA of FC rats, both in the presence and absence of nociceptive tone, suggesting a possible link of PPAR signalling and basolateral amygdalar dopaminergic modulation of fear and anxiety responses. My findings are in agreement with a recent study that have shown that PPARα-KO have enhanced fear learning compared to WT counterparts, and that this enhancement is associated with increased levels of dopamine in the amygdala (Chikahisa et al., 2019). Other studies have proposed that PPARs modulate dopamine signalling. Mijangos-Moreno et al. (2016) have shown that WY14643 (PPARα agonist) injected into the hypothalamus increased dopamine levels in the nucleus accumbens. This same agonist and methOEA (a long lasting form of OEA), when systemically administered, dose-dependently decreased nicotine-induced excitation of dopamine neurons in the VTA and nicotine-induced elevations of dopamine levels in the nucleus accumbens shell of rats (Mascia et al., 2011). Thus, we hypothesize that the blockade of PPARα in the BLA of FC rats affects dopamine signalling within this region, resulting in a delay in extinction learning. Moreover, the blockade of PPARγ in FC rats affects dopamine signalling in the BLA, which in turn may result in enhancement of the recall of fearful memories (Li et al., 2010). Alternatively, the blockade of these receptors may have affected AEA action on fear expression and/or extinction. Previous work from our group has shown increased levels of AEA in the BLA of FC rats that received intra-plantar formalin injection in the hind paw compared to NFC counterparts, and trends were also present for the other two NAEs - PEA and OEA (Rea et al., 2013). Recently, Morena et al. (2018) have demonstrated that the overexpression of FAAH in the BLA decreased expression of conditioned fear in the extinction training session and anxiety-related behaviour in rats. We hypothesize that AEA in the BLA may modulate fear processing through PPARα and PPARγ. Thus, the blockade of these receptors may have affected AEA action on fear expression and/or extinction. However, a possible role of PEA and OEA in this modulation cannot be disregarded. Further studies focusing on the activation of PPARs and the role of PEA and OEA signalling in the BLA in conditioned fear and anxiety could contribute to a better understanding of the role of PPAR signalling in the BLA in conditioned fear and anxiety.

Recent studies have pointed to a possible role of PPARs in anxiety. Youssef et al. (2019) have shown that the administration of a PPARγ antagonist blocked the anxiolytic effect of beta-caryophyllene. Another study demonstrated that repeated stress decreased

PPARγ expression in the amygdala, and treatment with anxiolytics recovered PPARγ expression (Liu et al., 2018). PPARγ blockade or knockout was shown to have anxiogenic effects on mice (Domi et al., 2016). In this same study, intra-amygdala injections of pioglitazone (PPARγ agonist) were shown to reduce stress-induced anxiety behaviour in rats. In our study, NFC rats that received intra-BLA injections of PPAR antagonists in the absence of nociceptive tone had increased levels of freezing, comparable to their FC counterparts. Thus, the blockade of these receptors in the BLA increased innate anxious state in NFC rats with absent formalin-evoked pain. The studies to-date have investigated the role of PPARs in provoked anxious state (i.e. stress or pharmacological-induced anxiety state). Our results support and extend these studies, demonstrating that PPAR signalling in the BLA may modulate anxiety-related behaviour in the absence of nociceptive tone.

The results suggest that PPAR signalling in the BLA does not mediate or modulate formalin-evoked nociceptive behaviour. As previously discussed in Chapters 1 and 2, other studies have demonstrated effects of PPAR agonists on pain-related behaviour (Taylor et al., 2002; Oliveira et al., 2007; Suardíaz et al., 2007; Gill et al., 2013, Mansouri et al., 2017) but less is known about the effect of PPAR antagonists. The exogenous administration of PPAR natural ligands has also been shown to modulate pain responses (see Okine et al. (2018) for a review). To our knowledge, the study described in the present chapter is the first study to investigate the effect of the blockade of PPARα, PPARβ/δ and PPARγ expressed in the BLA on inflammatory pain. Similarly to what was shown by Donvito et al. (2017) and Mansouri et al. (2017) in their systemic studies, and my own systemic studies in Chapter 2, PPAR antagonist administration into the BLA do not affect formalin-evoked nociceptive behaviour.

FCA is a potent suppression of nociceptive responses upon exposure to a fearful stimulus. The experiments described in this chapter investigated the effects of intra-BLA administration of PPAR antagonists on FCA. FCA has been previously shown to be associated with increased levels of AEA, an endocannabinoid which also binds to PPARs, in the BLA (Rea et al., 2013) and a strong trend for increased tissue levels of PEA and OEA, endogenous ligands of PPARs, in the BLA. No FCA-related alterations in the levels of AEA, PEA and OEA in the BLA was seen in my experiment; however, Rea et al (2013) used a different rat strain (Lister-hooded *versus* Sprague-Dawley) and a shorter trial compared to my experiment (15 minutes *versus* 30 minutes) which may explain the different observations in our studies. The data demonstrate that fear conditioning profoundly reduces formalin-

evoked nociceptive behaviour via FCA as we and others have shown previously (Roche et al., 2010; Ford et al., 2011; Rea et al., 2011, 2014; Butler et al., 2012; Olango et al., 2012) and that the blockade of PPARα, PPARβ/δ or PPARγ in the BLA does not affect expression of FCA.

Furthermore, we compared the effects of intra-plantar injection of formalin or saline on tissue levels of neurotransmitters, endocannabinoids and NAEs in the BLA of FC and NFC rats. The presence or absence of formalin-induced inflammatory pain was shown to influence the changes in the levels of neurotransmitters and NAEs after fear conditioning rats that received intra-BLA vehicle. For example, FC rats that received saline injection into the right hind paw had increased serotonin and AEA levels in the right BLA, but these effects were not observed in formalin-treated animals. Fear conditioning also decreased PEA levels in the right BLA of formalin-, but not saline-, treated rats. Rea et al (2013) have shown that PEA levels were higher in the left BLA of FC formalin compared to NFC formalin-treated counterparts, which is opposite to what we have observed in our experiment, in which PEA levels were higher in the right BLA of FC formalin compared to NFC formalin-treated counterparts. In addition, Rea et al (2013) did not observe altered AEA levels in the left or right BLA of saline-treated animals, contrary to what we have observed in our study, in which AEA levels were higher in FC saline-treated compared to NFC saline-treated rats. However, the rats used by Rea et al (2013) were from a different strain (Lister-hooded versus Sprague Dawley) which may explain the difference in the results between our studies. The higher levels of serotonin in FC saline-treated rats compared to NFC counterparts that we have observed are in accordance to what Zanoveli et al (2009) have seen in their microdialysis study. Similarly, serotonin levels were increased in conditioned rats 30 minutes after re-exposure. Other studies have also shown that serotonin neurotransmission in the BLA can be involved in the facilitation of conditioned states (Davis et al., 1994; Deutch and Charney, 1996; Macedo et al., 2007). Formalin-injection itself affected dopamine and AEA levels. NFC rats which received an intra-plantar formalin injection were shown to have increased levels of both dopamine and AEA in the right BLA compared to their saline-treated counterparts. Although the role of dopaminergic signalling in pain responses has been extensively studied (Wood, 2008; Ikeda et al., 2014; Benarroch, 2016; Taylor et al., 2016) the role of this system in the amygdala in nociception is less examined. Roche et al (2007) did not find changes in dopamine levels in the amygdaloid complex of formalin-treated rats compared to saline-treated rats, which contrast with our findings. The

rats, similarly to our study, underwent cannula implantation into the BLA but were only re-exposed for 15 minutes to the arena. Additionally, the levels of dopamine were measured using a different technique (i.e. HPLC with electrochemical detection) in gross dissected amygdala in contrast with punches of each of the amygdalar nuclei separately. One study demonstrated that antagonism of D_1 receptors in the BNST (part of the extended amygdala) enhanced nociceptive responses in female, but not male rats, suggesting that the dopaminergic system in the BNST may exert sexually dimorphic effects on pain (Hagiwara et al., 2013). The blockade of dopaminergic receptors in the nucleus accumbens prevented antinociceptive effects of CB_1 receptor activation in the BLA, suggesting a link between neuromodulation of pain in the BLA and the mesolimbic dopaminergic system. The blockade of D_2 and D_4 in the PFC inhibited long lasting suppression of nociceptive responses induced by high frequency stimulations of the BLA, suggesting a link between neuromodulation of pain and the prefrontal dopaminergic system. In their investigation, Rea at al (2013) did not see any changes in AEA levels in the BLA of formalin-treated rats compared to saline-treated counterparts, which is divergent to what we have observed in our results. However, their re-exposure time to the conditioning arena was longer (45 or 60 min) than the one used in our experiments (30 min) which may account for this difference. In our experiment, FC formalin-treated rats had higher levels of PEA in the right BLA compared to saline-treated animals, a result also seen by Rea et al (2013). Together, these results show that intra-plantar formalin injection impacts neurotransmitters and NAE signalling in the BLA. Thus, it is possible that these neurochemical differences underpin the differential effects of PPAR blockade on conditioned fear-related behaviour in the presence versus absence of formalin-evoked nociceptive tone.

In conclusion, these studies have shown that the blockade of PPARα expressed in the BLA impaired short-term, within trial fear-extinction, and the blockade of PPARγ in the same region potentiated freezing expression in the presence of a nociceptive stimulus in rats, without affecting pain responses. Moreover, the blockade of PPARα, PPARβ/δ and PPARγ in the BLA increased innate anxiety status in the absence of pain in NFC rats. These results indicate a possible modulatory role for PPARs in the BLA in fear/anxiety expression, with differential effects depending on the presence or absence of nociceptive tone. Further investigations are necessary to elucidate the possible mechanisms involved in these modulations and clarify the molecular basis on this differential pain-dependent effect.

It still remained unclear if these effects were exclusive to the BLA nuclei or if other regions of the amygdala can also be affected by PPAR signalling manipulations. In order to explore the possibility of the participation of PPARs from other amygdalar regions on conditioned fear, FCA, and inflammatory pain-related behaviour I conducted two additional studies, in which I investigated the effects of the blockade of PPARs expressed in the CeA on FCA and formalin-evoked nociceptive behaviour, which will be viewed in detail in the next chapter. I also examined if the blockade of PPARs in the CeA would differentially affect conditioned fear expression and/or extinction in the presence versus absence of formalin-evoked nociceptive tone.

Chapter 4: Effects of intra-CeA administration of PPAR antagonists on formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence or absence of nociceptive tone in rats

4.1 Introduction

In the previous chapter, we have shown that the blockade of PPARs expressed in the BLA has different effects on fear behaviour depending on the presence or absence of pain. Specifically, the data revealed that the blockade of PPARα and PPARγ in the BLA, in the presence of nociceptive tone, prolongs freezing in FC animals. Also, the blockade of the three isoforms in the BLA, in the absence of nociceptive tone, increased freezing in NFC rats. However, the question remains as to whether these results are exclusive to the BLA or if PPARs expressed in other subnuclei of the amygdala may also mediate or modulate fear responses. In the present chapter, I investigated if the effects of PPAR blockade seen in the BLA can also be observed in another subnucleus of the amygdala which has been shown to be equally important to fear responses, the CeA.

The CeA is one of three groups of nuclei in the amygdala, according to the nomenclature proposed by Price (see section 1.3.3.1). It is differentiated from the other two groups on account of its connections, embryonic origin and cytoarchitecture (Sah et al., 2003). Formerly, the CeA was seen as a homogeneous structure that served as an output of fear responses. However, recent research is demonstrating that the region is not that simple. It is widely accepted that its medial and lateral portions are anatomically and functionally different and have different contributions in the fear circuitry, although the intra-CeA circuitry is not completely understood (Sah et al., 2003). The CeA receives input from several brain regions, including the lateral (LA), basolateral (BLA) and basomedial (BMA) amygdala, dysgranular and agranular insula, infralimbic cortex (IF), bed nucleus of the stria terminalis (BNST), pontine parabrachial nucleus, auditory cortex and auditory thalamus (Sah et al., 2003; LeDoux, 2007; Keifer et al., 2015b). It also has an extensive efferent network, which includes the lateral (LH), paraventricular nucleus (PVN), and dorsomedial hypothalamus (DMH), PAG, medial preoptic area, and other many indirect connections (Sah et al., 2003; Keifer et al., 2015b). Lesions (Roozendaal et al., 1990, 1991; Helmstetter, 1992a; Campeau and Davis, 1995; Killcross et al., 1997; Goosens and Maren, 2001; Nader et al., 2001; Choi and Brown, 2003; Koo et al., 2004; Zimmerman et al., 2007) or inactivation (Fanselow and Kim, 1994; Walker and Davis, 1997; Goosens and Maren, 2003;

Wilensky et al., 2006; Zimmerman et al., 2007; Rabinak et al., 2009) of the CeA impair acquisition and expression of conditioned fear. Moreover, there is an increase in markers of synaptic plasticity within the CeA in response to fear conditioning (Samson et al., 2005). Hence, our understanding of the role of the CeA in fear responses has evolved, and the region is no longer viewed as only an output for fear responses, but as a region with potential independent modulatory role in fear expression.

As pointed out in Chapter 2, there is a paucity of information on the role of PPARs expressed in the BLA or CeA in fear or anxiety. There is some evidence that PPARγ blockade or neuronal knockout has anxiogenic effects on mice (Domi et al., 2016; Youssef et al., 2019), however systemic PPARα antagonism did not reverse the anxiolytic effect of a FAAH inhibitor (Danandeh et al., 2018). Whether PPARβ/δ modulates anxiety or fear remains unexplored. Moreover, the specific role of PPARα, PPARβ/δ and PPARγ expressed in the CeA in anxiety or fear responses remains to be investigated.

Pain is a complex condition with sensory-motor, emotional and cognitive aspects. The amygdala is part of both the descending pain pathway and the limbic system, and is involved in the emotional-affective aspect of pain responses. The CeA in particularly is important in pain processing (Neugebauer et al., 2004, 2009; Veinante et al., 2013; Neugebauer, 2015; Thompson and Neugebauer, 2017). Neuronal activity in the CeA is increased in several models of pain (Veinante et al., 2013), including the formalin model (Carrasquillo and Gereau, 2007, 2008; Butler et al., 2017). Studies using electrophysiological (Bernard et al., 1992; Neugebauer and Li, 2002) and optogenetic (Sugimura et al., 2016) approaches have characterized the responses of CeA neurons to noxious stimuli. Additionally, as mentioned already, the CeA is highly connected with nociceptive centres such as PAG, hypothalamus, and parabrachial nucleus (Sah et al., 2003; Neugebauer et al., 2004; Veinante et al., 2013; Thompson and Neugebauer, 2017), especially its capsular subdivision (CEC), which is called the "nociceptive amygdala". Therefore, changes in neuronal activity as a consequence of nociceptive information coming from the thalamus (thalamus-BLA-CeA) or parabrachial nucleus (PB-CeA) pathways modulates nociceptive responses.

As discussed previously, pain and fear modulate one another in a reciprocal manner. The phenomenon known as fear-conditioned analgesia (FCA), in which a fearful stimulus causes a significant reduction in pain response, is an example of the influence of fear on pain. Similarly, pain can regulate fear responses. Post-traumatic stress disorder (PTSD) symptoms

tend to be more pronounced in patients with chronic pain (Asmundson et al., 2002). Moreover, patients with chronic pain are twice as likely to develop phobias (Pereira et al., 2017). PPAR isoforms are expressed in brain regions that play an important role in pain and fear/anxiety such as the amygdala (Warden et al., 2016), PFC (Moreno et al., 2004; Okine et al., 2014; Warden et al., 2016), hippocampus (Moreno et al., 2004; Domi et al., 2016) and PAG (Okine et al., 2017).

PPARs mRNA and protein has been shown to be expressed in the amygdala (Warden et al., 2016). However, potential differences in the expression of these receptors in the distinct subnuclei of the amygdala have not yet completely determined. I have presented in chapter 2 evidence of PPAR expression in the BLA and, in the present chapter, I aim to verify the expression of these receptors in the CeA.

In this chapter, I investigated the hypothesis that the blockade of PPARs expressed in the CeA decreases tonic, persistent inflammatory pain and increases conditioned fear. Specifically, I examined the effects of intra-CeA administration of GW6471 (PPARα antagonist), GSK0660 (PPARβ/δ antagonist), and GW9662 (PPARγ antagonist) on formalin-induced nociceptive behaviour and FCA in rats. I also investigated the effects of intra-CeA administration of these antagonists on conditioned-fear related behaviour both in the presence and absence of formalin-evoked nociceptive tone in rats. In addition, associated changes in tissue levels of neurotransmitters, endocannabinoids, and NAEs in the CeA were analysed. Furthermore, differences in the levels of neurotransmitters, endocannabinoids and NAEs in the CeA of FC and NFC rats that received either intra-plantar formalin or saline injection were also analysed. Therefore, the specific aims of the studies described in this chapter were:

- To verify the expression of the three subtypes of PPARs in the rat CeA through western blotting and RT-qPCR techniques.
- To determine if PPAR signalling within the CeA plays a role in expression of tonic, persistent inflammatory pain and FCA by examining the effects of intra-CeA administration of PPAR antagonists on formalin-evoked nociceptive behaviour and FCA in rats, and associated alterations in levels of neurotransmitters, endocannabinoids, and NAEs in the CeA.
- To determine if PPAR signalling within the CeA plays a role in the expression of conditioned fear in the presence and in the absence of nociceptive tone by examining

the effects of intra-CeA administration of PPARs antagonists on fear-related behaviour, and associated alterations in levels of neurotransmitters, endocannabinoids, and NAEs in the CeA.

- To determine if the presence of nociceptive tone influences the levels of neurotransmitters, endocannabinoids and NAEs in NFC and FC rats through a comparison of their levels in formalin and saline-treated rats that received vehicle microinjections into the BLA and CeA .
- To investigate if the fear-related behavioural changes after blockade of PPARα, PPARβ/δ and PPARγ seen in the previous chapter 3 are exclusive to the BLA or if other nuclei in the amygdala, particularly the CeA, contribute to the effects observed previously.

4.2 Materials and Methods

4.2.1 Animals

Experiments were carried out on a total of 92 (Experiment 1) and 90 (Experiment 2) adult male Sprague-Dawley rats (230-250g on arrival; Envigo UK, Bicester, England). The animals were maintained at controlled temperature (22 ± 2°C) and humidity (45-55%) under standard lighting conditions (12:12h light-dark cycles, lights on from 07.00). Animals were housed 2-3 per flat bottomed cage (L:45 x H:20 x W:20cm) containing 3Rs paper bedding material (Fibrecycle Ltd., North Lincolnshire, United Kingdom) and sizzle nest material (LBS Biotechnology, Horley, United Kingdom) for the first week after arrival, and were posteriorly singly housed after surgery and for the rest of the experiment. 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.

4.2.2 Cannula Implantation

Under isoflurane anaesthesia (2-3% in O_2, 0.7L/min), a stainless steel guide cannula (12mm length, Plastics One Inc., Roanoke, Virginia, USA) was stereotaxically implanted 1mm above the right and left CeA of each rat (coordinates: AP = -2.5mm from bregma, ML = ±4.6mm, DV = -7.2mm from the skull surface) according to the rat brain atlas published by Paxinos and Watson, 1997 (Paxinos et al., 1997). The cannulae were permanently fixed to the skull using stainless steel screws and carboxylate cement. A stylet made from stainless steel tubing (12mm length, 22G, Plastic One – Bilaney Consultants, Sevenoaks, UK) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (1.25mg/25µL, s.c., Rimadyl, Pfizer, Kent, UK), was administered before the surgery to manage postoperative analgesia. Animals received a single daily dose of the antimicrobial agent enrofloxacin (10mg/kg, s.c., Batyril, Bayer plc, Berkshire, UK) for 5 days to prevent postoperative infection. Following cannula implantation, the rats were singly housed and at least 6 days were allowed for recovery post-surgery prior to experimentation. During this recovery period, the rats were handled, stylets checked, and their body weight and general health monitored once daily.

4.2.3 Drugs

PPARα antagonist, GW6471, PPARβ/δ antagonist, GSK0660, and PPARγ antagonist, GW9662 (all obtained from Tocris Bioscience, Bristol, UK) were dissolved in a 100% Dimethyl sulfoxide (DMSO), used as vehicle solution. The antagonist doses were identical to those used in the studies described in Chapter 3. The dose of GW6471 (10μg/0.5μl) was chosen based on a study showing that this dose delayed the onset of the second phase of formalin-evoked nociceptive behaviour (Okine et al., 2014). The dose of GW9662 (10μg/0.5μl) was chosen based on a previous study showing that this dose was effective in reversing the anti-inflammatory and anti-hyperalgesic actions of rosiglitazone (Morgenweck et al., 2010). We used the same dose of GSK0660 (10μg/0.5μl) as that used for the other two antagonists for comparison, because no published studies have administered this drug intracerebrally. Formalin was prepared from a 37% stock solution (Sigma-Aldrich, Dublin, Ireland) diluted in 0.9% sterile saline. Sodium chloride was dissolved in distilled water (9g in 1L – 0.9%) and the solution was autoclaved to avoid infections and inflammation.

4.2.4 Experimental Procedure

Two different experiments using two different cohorts of rats were carried out (Experiments 1 and 2) and were identical in design and methodology with the exception that rats in Experiment 1 received intra-plantar injection of formalin while those in Experiment 2 received intra-plantar injection of saline. The FCA paradigm used in both experiments was essentially as described before (Finn et al., 2004; Butler et al., 2008; Rea et al., 2018) and in Chapter 2 (Section 2.2.2). There were two phases: conditioning (day 1) and test (day 2). On the conditioning day, rats were placed in a Perspex chamber (30 cm x 30 cm x 40 cm) and after 15 seconds they received the first of 10 footshocks (0.4mA, 1se duration, LE85XCT Programmer and Scrambled Shock Generator; Linton Instrumentation, Norfolk, UK) spaced 60s apart. Fifteen seconds after the last footshock, rats were returned to their home cage. The animals that belonged to the control group, that did not receive footshocks, were placed in the chamber for an equivalent time (9min45sec). The animals were randomly assigned to one of 8 groups – rats that received footshocks (FC) or no footshocks (NFC) treated with GW6471, GSK0660, GW9662, or vehicle (100% DMSO).

The test day started 23hrs 30min after the end of the conditioning phase (Figure 4.1). First, the rats received a 50μl injection of formalin (2.5% in 0.9% saline; Experiment 1) or

saline (0.9%; Experiment 2) into the right hind paw under brief isoflurane anaesthesia (3% in O_2; 0.8L/min). Fifteen minutes after, the animals received intra-central nucleus of the amygdala (intra-CeA) microinjections of either the PPARα antagonist (GW6471), the PPARβ/δ antagonist (GSK0660), PPARγ antagonist (GW9662) or vehicle (volume of injection 0.5µl/side). After the administrations, the rats were returned to their home cages. Fifteen minutes after microinjections, or 24 hours after footshock, the rats were re-exposed to the conditioning chamber. A video camera located beneath the observation chamber was used to monitor animal behaviour for 30min. For this experiment, it was decided that 30min duration re-exposure was more adequate to observe changes in FCA. At the end of the test phase (60 min post-formalin injection), rats were killed by decapitation, fast-green dye injected via the guide cannulae (see below), brains were removed, snap-frozen on dry ice and stored at −80°C. Formalin-induced oedema was assessed by measuring the change in the diameter of the right hind paw immediately before, and 60min after, formalin administration, using Vernier callipers.

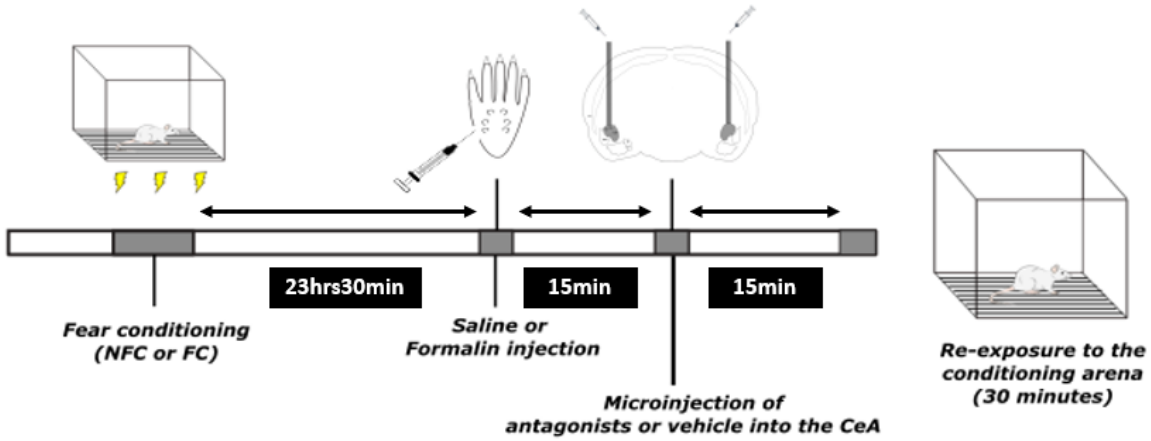


Figure 4.1: Graphical representation of the experimental procedure.

4.2.5 Behavioural analysis

See Chapter 2, section 2.2.4

4.2.6 Brain extraction

See Chapter 2, section 2.2.5

4.2.7 Histological verification of intracerebral injection sites

Stereotaxic coordinates were verified histologically on 4 animals before the start of the cannula implantation surgeries. The rats underwent the surgical procedure detailed in the section 4.2.2. After the conclusion of the surgical implantation of cannulae, the 4 rats, still under anaesthesia, were decapitated and a microinjection of 2% fast green dye (0.5µl over 1 minute; Sigma-Aldrich, Dublin, Ireland) diluted in DMSO was made to decide if the coordinates used were accurate for the CeA. The brain was collected and snap-frozen on dry ice. Then, frozen coronal brain sections were cut at 50 µm thickness on a cryostat at -21°C from the start to the end of the amygdaloid complex to determine the location of the dye and confirm coordinates. For all other rats in the experiments, the dye injection was performed immediately post-decapitation in order to determine if the injections successfully in targeted the CeA.

4.2.8 Cryo-sectioning and tissue microdissection

Frozen coronal brain sections of 150µm thickness containing the central nucleus of the amygdala (CeA) were cut on a cryostat (Leica Biosystems, Watznal, Germany), and were punch-dissected as previously described (Ford et al., 2008; Olango et al., 2012a; Rea et al., 2014) using cylindrical brain punchers (Harvard Apparatus, MA, USA) with an internal diameter of 0.50mm at the following rostro-caudal levels (obtained from the rat brain atlas by Paxinos and Watson, 2006: (CeA) Bregma, - 2.32 – -3.30mm. Additionally, in order to evaluate possible lateralisation effects, the CeA punches were separately collected for right and left hemispheres. The punch-dissected tissue was weighed (2.1±0.3mg) and stored at -80°C prior to measurement of AEA, PEA, OEA, 2-AG and neurotransmitter levels by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

4.2.8 Measurement of NAEs and neurotransmitters in discrete brain regions using liquid chromatography – tandem mass spectrometry (LC-MS/MS)

See Chapter 2, section 2.2.7

4.2.9 Verification of PPAR expression in the CeA

4.2.9.1 Verification of PPAR expression in the CeA by Western blotting

The method was identical to that described in section 3.2.9.1 with the exception that punched CeA tissue from naïve male Sprague Dawley rats was used.

3.2.9.2 Verification of PPARβ/δ expression in the CeA by RT-qPCR

The method was identical to that described in section 3.2.9.2 with the exception that punched CeA tissue from naïve male Sprague Dawley rats was used.

4.2.10 Statistical Analysis

The SPSS 22.0 statistical package was used to analyse data. Normality was assessed using Shapiro-Wilk test and homogeneity of variance was checked using Levene's test. Behavioural data were analysed using two-factor analysis of variance (Two-way ANOVA), with factors being fear-conditioning and treatment, or repeated measures ANOVA when appropriate (e.g. when the data were analysed and presented in time bins). Neurochemical data were analysed using three-factor analysis of variance (Three-way ANOVA), with factors being fear conditioning, treatment, and side (ipsilateral or contralateral, with respect to the formalin injection). *Post hoc* pairwise comparisons were made with Student Newman-Keuls test when appropriate. If data were found to be non-parametric, three transformation were applied, in this order: square root of the data values, log of the data values, and ranking of the data values. Also, it was checked if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular data set being analysed (Thunder et al., 2007). If data were still deemed non-parametric after these transformations and tests, they were analysed using Kruskal-Wallis analysis of variance and *post hoc* analysis performed using Dunn's test when appropriate. When repeated measures were non-parametric distributed, data were analysed using Friedman's and Kruskal Wallis tests followed by Dunn's *post hoc* test if applicable. Data were considered significant when $p<0.05$. Data are expressed as group means ± standard error of the mean (S.E.M.) when parametric and as median with interquartile range and min/max values when non-parametric. Possible presence of outliers was checked by assessing the distribution of data. In case the data 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.

4.3 Results

4.3.1 Experiment 1: Effects of intra-CeA administration of PPAR antagonists on formalin-evoked nociceptive behaviour, FCA and conditioned fear in the presence of nociceptive tone in rats

4.3.1.1 Histological verification of microinjection sites

After histological verification, 76% of the rats had both injections correctly placed within the borders of both CeA. Also, 7% had one of the injections in the BLA and the other outside BLA borders. The remaining 17% were placed in the BLA, BMA, or internal capsule. The data analysed were derived only from rats where intracerebral microinjections were accurately placed in the CeA (Figure 4.2).

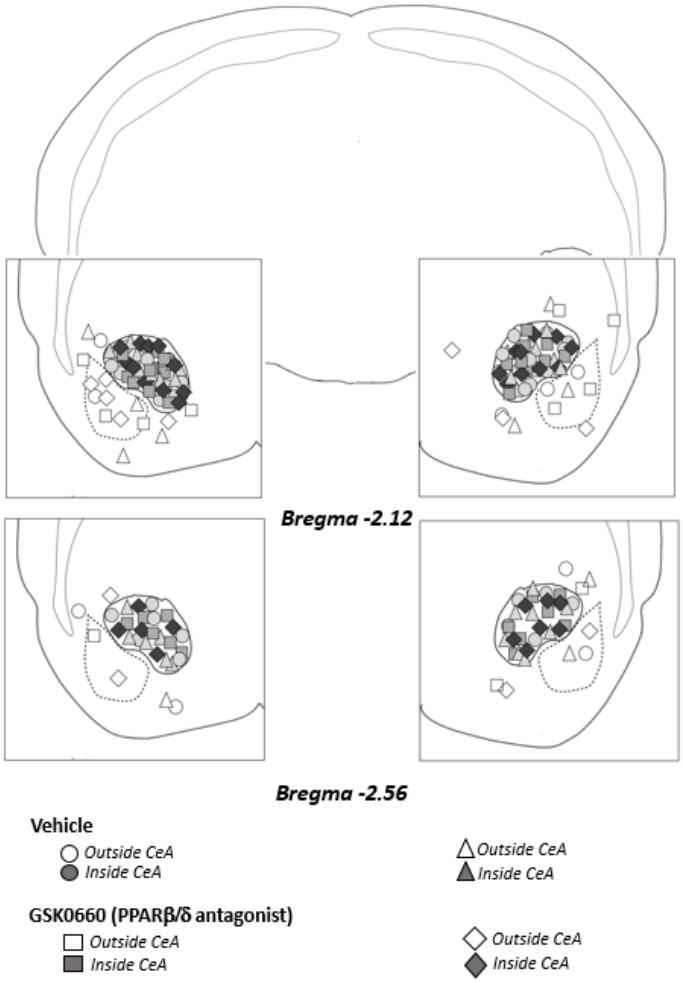


Figure 4.2: Histological verification of injector site location.

4.3.1.2 Intra-CeA administration of GW6471, GSK0660, and GW9662 had no effects on formalin-evoked nociceptive behaviour or FCA

Intra-plantar administration of formalin into the right hind paw produced robust nociceptive behaviour as evidenced by the composite pain score. Two-way ANOVA revealed an effect of fear conditioning [F (1, 56) = 4.741, $^{a}p<0.05$] on pain behaviour (Figure 4.3). However, *post hoc* testing with Student Newman-Keuls test did not reveal any significant differences between groups. There were no significant effects of treatment [F (3, 56) = 0.0408, $p>0.05$], or treatment x conditioning [F (3, 56) = 1.425, $p>0.05$] on CPS values. Two-way ANOVA revealed no significant effect of fear-conditioning [F (1, 70) = 0.5964, $p>0.05$], treatment [F (3, 70) = 1.879, $p>0.05$], or treatment x conditioning [F (3, 70) = 1.121, $p>0.05$] on formalin-induced paw oedema (Figure 4.4).

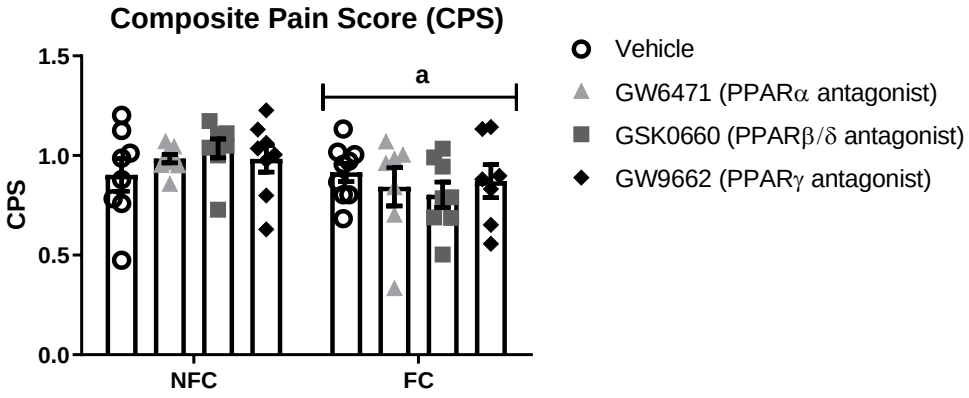


Figure 4.3: Effects of intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on formalin-evoked nociceptive behaviour in non-fear conditioned (NFC) and fear conditioned (FC) rats. Two-way ANOVA indicated a significant overall effect of fear conditioning ($^{a}p<0.05$). Data are expressed as mean ± S.E.M (n=7-10 rats per group).

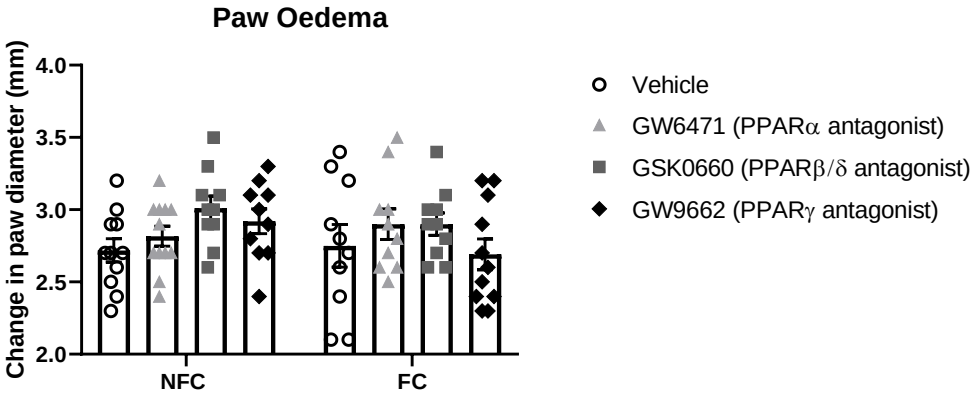


Figure 4.4: Effects of intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on formalin-evoked hind paw oedema in non-fear conditioned (NFC) and fear conditioned (FC) rats. Paw oedema was assessed by measuring the change in the diameter of the right hind paw immediately before, and 60 min after, formalin administration. Data are expressed as mean ± S.E.M (n=7-10 rats per group).

4.3.1.3 Intra-CeA administration of GW6471, GSK0660, and GW9662 had no effects on fear-related behaviour in formalin-treated rats

Two-way ANOVA revealed a significant effect of fear conditioning [$F(1, 56) = 33.064$, $^{a}p<0.001$] on freezing duration. *Post hoc* test with Student Newman-Keuls indicated that FC rats treated with GSK0660 and GW9662 had increased freezing duration compared to their NFC counterparts ($^{*}p<0.05$ vs NFC GSK0660, $^{*}p<0.05$ vs NFC GW9662). There were no significant effects of treatment [$F(3, 56) = 2.446$, $p=0.073$] or the interaction of treatment x fear conditioning [$F(3, 56) = 0.954$, $p>0.05$] on freezing duration. When each conditioning group were analysed separately, one-way ANOVA revealed a trend for a treatment effect on the NFC group [$F(3, 33) = 2.799$, $p=0.058$] but not in the FC group [$F(3, 31) = 1.365$, $p>0.05$] (Figure 4.5A).

Friedman's test revealed an effect of time on freezing duration [$\chi^2(9) = 255.987$, $p<0.001$]. Kruskal Wallis test revealed a significant difference among groups in the first 3 min [$Time_{1\text{-}3}\ \chi^2(7) = 24.673$, $p<0.01$]. *Post hoc* analysis with Dunn's test did not reveal any significant difference among groups. When each conditioning group is analysed separately, Friedman's test revealed an effect of time on NFC [$\chi^2(9) = 141.197$, $p<0.001$] and FC [$\chi^2(9) = 125.066$, $p<0.001$] rats. Kruskal Wallis analysis did not reveal significant differences among groups in NFC or FC in any of the time bins (Figure 4.5B).

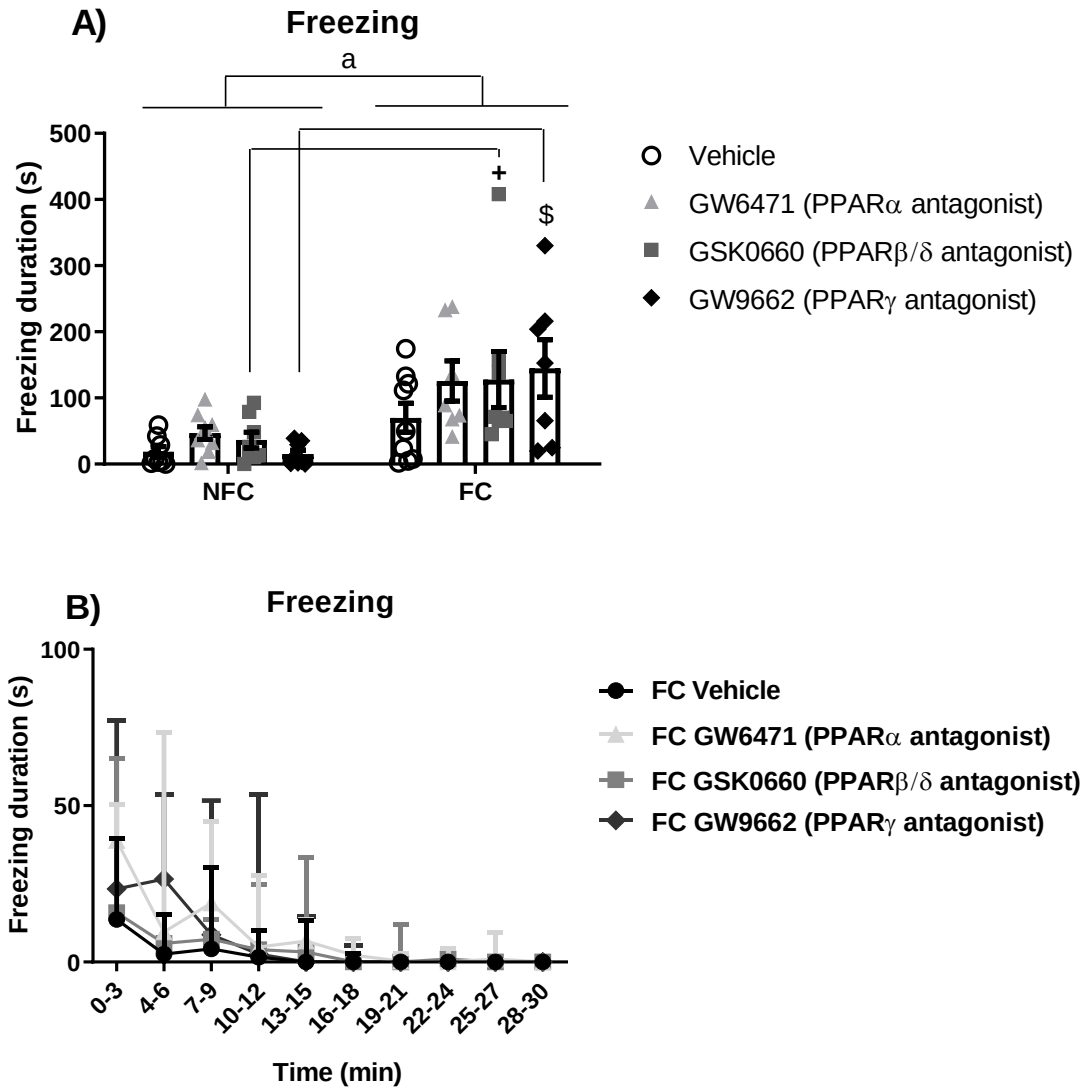


Figure 4.5: Effects of fear conditioning and intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on total (A) and 3-min bins (B) freezing duration in NFC and FC rats that received intra-plantar injection of formalin. Two-way ANOVA revealed a significant effect of fear conditioning ($^{a}p<0.001$) on freezing duration. Post hoc analysis revealed a significantly higher duration of freezing in FC rats treated with GSK0660 and GW9662 compared to NFC counterparts ($^{+}p<0.05$ vs NFC GSK0660; $^{\$}p<0.05$ vs NFC GW9662). Data are expressed as means ± S.E.M. (n=7-10 rats per group).

Kruskal Wallis test revealed a significant difference among groups [χ^2 (7) = 24.106, p=0.001] in number of faecal pallets (Figure 4.6). However, *post hoc* analysis with Dunn's test did not show any significant pairwise differences between groups.

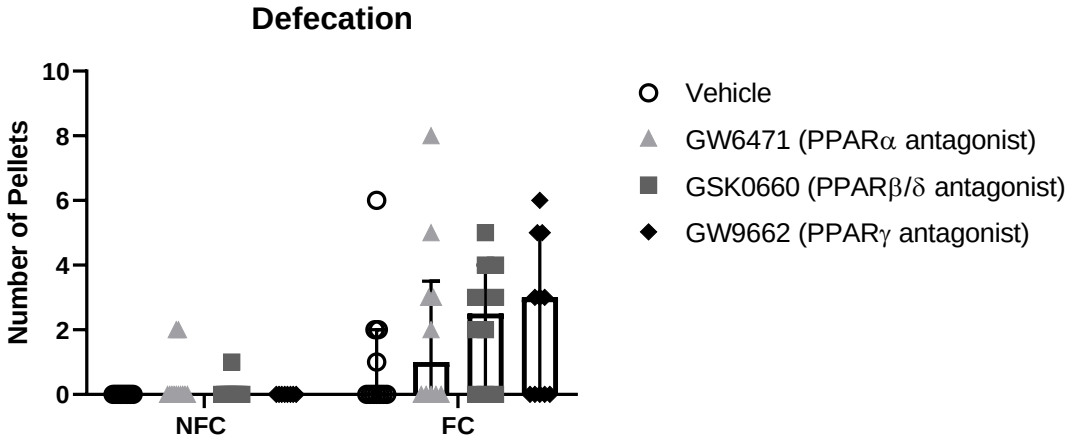


Figure 4.6: Effects of fear conditioning and intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on defecation. Data are expressed as median with interquartile range and min/max (n= 7-10 rats per group).

4.3.1.4 Intra-CeA administration of GW6471, GSK0660, and GW9662 had no effects on general/motor behaviour

Two-way ANOVA revealed a significant effect of treatment [F (3, 77) = 3.104, p<0.05] on walking duration (Figure 4.7A). However, *post hoc* analysis with Student Newman-Keuls did not show any significant differences between groups. There were no significant effects of fear conditioning [F (1, 77) = 2.156, p>0.05] or the interaction between treatment x fear conditioning [F (3, 77) = 1.141, p>0.05] on walking duration. When each conditioning group was analysed separately, one-way ANOVA did not show any significant effect of treatment in NFC [F (3, 43) = 2.263, p>0.05] or FC [F (3, 38) = 1.921, p>0.05] on walking duration.

Two-way ANOVA reveal that there were no significant effects of treatment [F (3, 56) = 0.592, p>0.05], fear conditioning [F (1, 56) = 0.307, p>0.05] or the interaction between treatment x fear conditioning [F (3, 56) = 1.138, p>0.05] on distance moved (Figure 4.7B). When each conditioning group was analysed separately, one-way ANOVA did not show any significant effect of treatment in NFC [F (3, 33) = 1.644, p>0.05] or FC [F (3, 33) = 0.196, p>0.05] on distance moved.

Kruskal Wallis test did not reveal any significant difference among groups [χ^2 (7) = 7.904, p>0.05] in rearing duration (Figure 4.7C). When each conditioning group was analysed separately, Kruskal Wallis test did not show any significant effect of treatment in NFC [χ^2 (3) = 4.702, p>0.05] or FC [χ^2 (3) = 2.154, p>0.05] on rearing duration.

Kruskal Wallis test did not reveal any significant difference among groups [χ^2 (7) = 7.541, p>0.05] in grooming duration (4.7D). When each conditioning group was analysed separately, Kruskal Wallis test did not show any significant effect of treatment in NFC [χ^2 (3) = 2.139, p>0.05] or FC [χ^2 (3) = 5.280, p>0.05] on grooming duration.

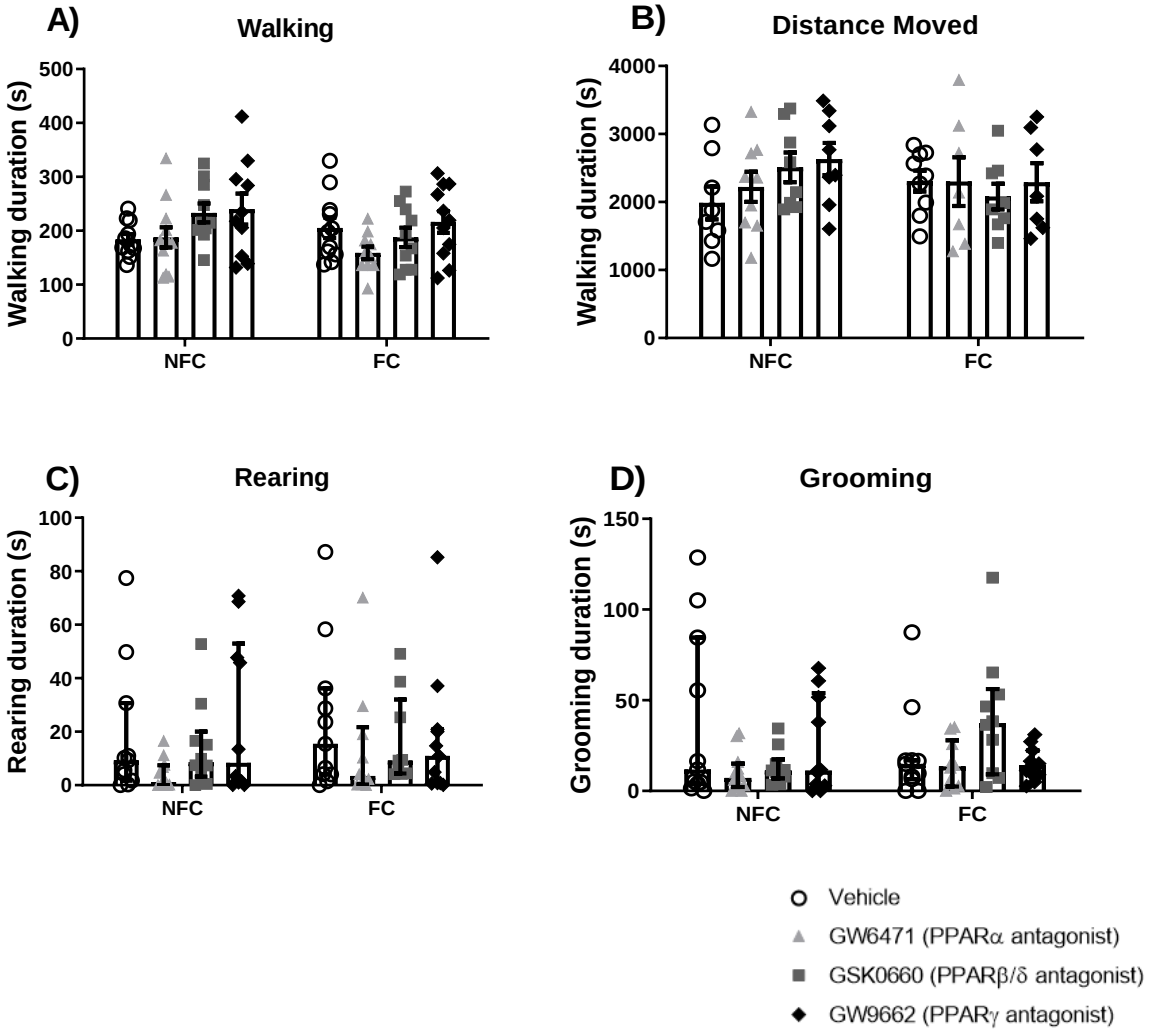


Figure 4.7: Effects of fear-conditioning and intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on walking duration (A), distance moved (B), grooming duration (C), and rearing duration (D). Data are expressed as mean ± S.E.M. (A, B) and median with interquartile range and min/max (C, D) n=7-10 rats per group.

4.3.2 Experiment 2: Effects of intra-CeA administration of PPAR antagonists on conditioned fear in the absence of nociceptive tone in rats

4.3.2.1 Histological verification of microinjection sites

After histological verification, 73% of the rats had both injections correctly placed within the borders of both CeA. Also, 5% had one of the injections in the BLA and the other outside BLA borders (Figure 4.8). The remaining 22% were placed in the BLA, BMA, or internal capsule. The data analysed were derived only from rats where intracerebral microinjections were accurately placed in the CeA.

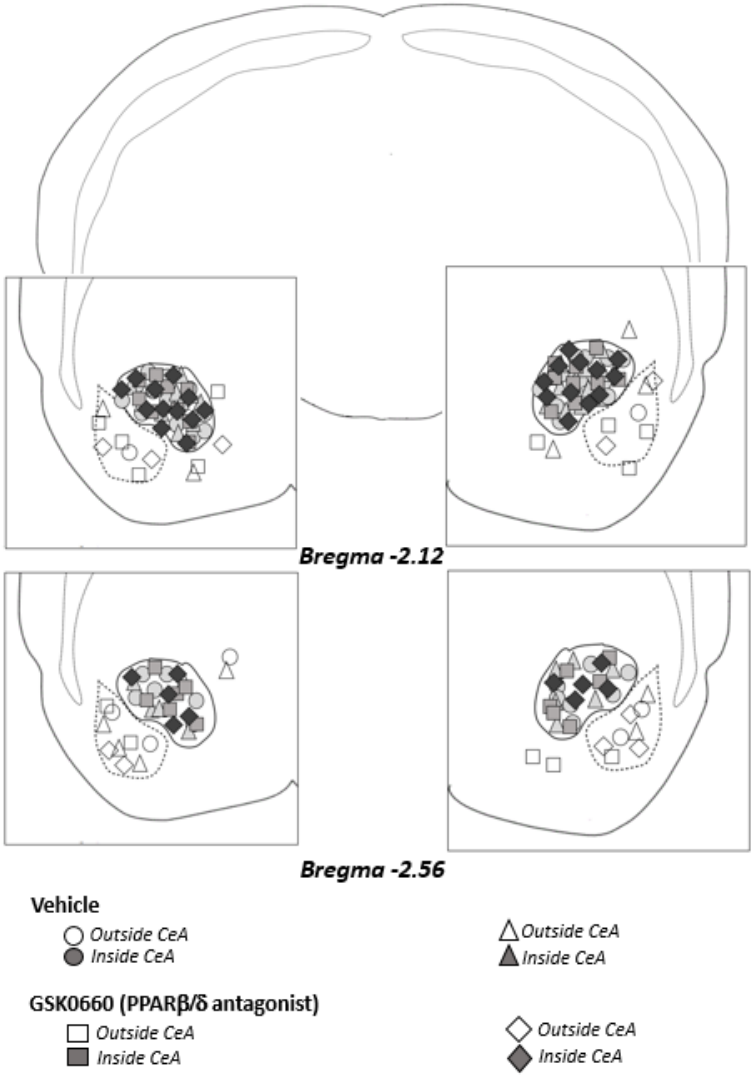


Figure 4.8: Histological verification of injector site location.

4.3.2.2 Intra-CeA administration of GW6471, GSK0660, and GW9662 had no effects on composite pain score or paw oedema in saline-injected rats

Composite pain scores were substantially less in this experiment following intra-plantar saline injection compared with Experiment 1 where rats received intra-plantar formalin injection (Figure 4.9). There were no significant effects of treatment [F (3, 76) = 1.210, p>0.05], fear conditioning [F (1, 76) = 0.049, p>0.05], and treatment x fear conditioning [F (3, 76) = 0.159, p>0.05] on CPS values.

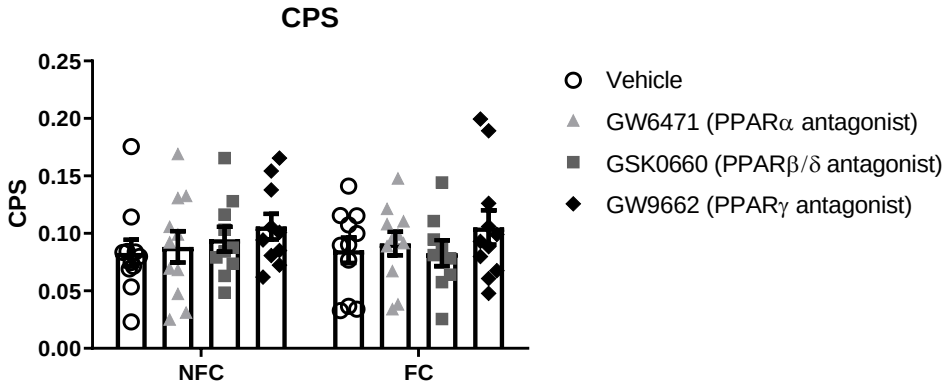


Figure 4.9: Effects of intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on composite pain score in NFC and FC rats that received intra-plantar injection of saline. Data are expressed as mean ± S.E.M (n=7-10 rats per group).

Change in paw diameter was substantially less in this experiment following intra-plantar saline injection compared with Experiment 1 where rats received intra-plantar formalin injection (Figure 4.10). There were no significant effects of treatment [F (3, 79) = 0.375, p>0.05], fear conditioning [F (1, 79) = 0.777, p>0.05], and treatment x fear conditioning [F (3, 79) = 0.856, p>0.05] on the paw diameter.

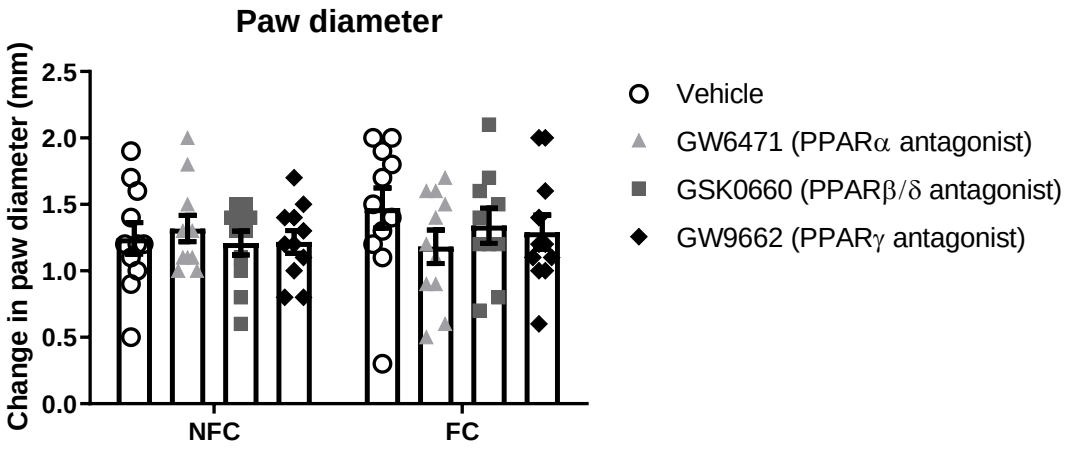


Figure 4.10: Effects of intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on the change in paw diameter in non-fear conditioned (NFC) and fear conditioned (FC) rats that received intra-plantar injection of saline. Data are expressed as mean ± S.E.M (n=7-10 rats per group).

4.3.2.3 Intra-CeA administration of GW6471, GSK0660, and GW9662 had no effects on fear-related behaviour in saline-treated rats

Two-way ANOVA revealed a significant effect of fear conditioning [$F(3, 63) = 8.577$, $^{aa}p<0.01$], but not of treatment [$F(1, 63) = 1.443$, $p>0.05$] or the interaction between treatment x fear conditioning [$F(3, 63) = 1.288$, $p>0.05$] on freezing duration (Figure 4.11). However, *post hoc* analysis with Student Newman-Keuls did not show any significant difference between groups.

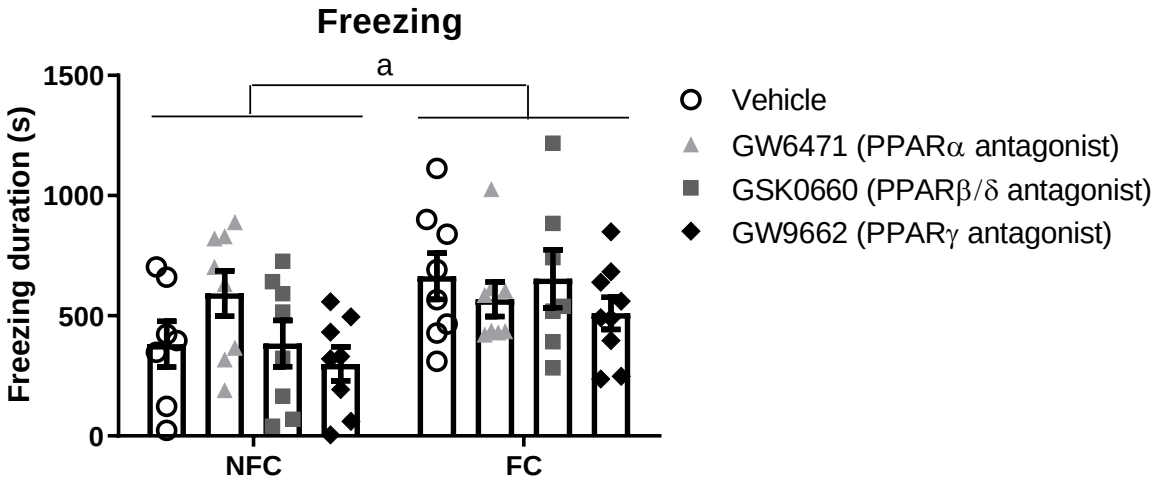


Figure 4.11: Effects of fear conditioning and intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on total freezing duration in NFC and FC rats that received intra-plantar injection of saline. Two-way ANOVA revealed an overall effect of fear conditioning ($^{a}p<0.01$). Data are expressed as mean ± S.E.M. (n=7-9 rats per group).

Kruskal Wallis test revealed a significant difference among groups [χ^2 (7) = 32.986, p<0.001] in number of faecal pallets (Figure 4.12). However, *post hoc* analysis with Dunn's test did not show any significant differences between groups.

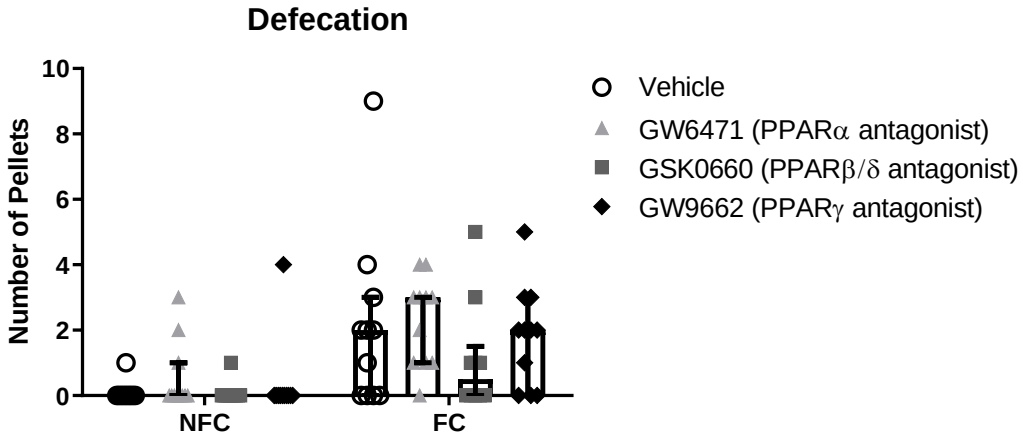


Figure 4.12: Effects of fear conditioning and intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on defecation. Data are expressed as median with interquartile range and min/max (n= 7-10 rats per group).

4.3.2.4 Intra-CeA administration of GW6471, GSK0660, and GW9662 had no effects on general/motor behaviour

There were no significant effects of treatment [F (3, 79) = 0.927, p>0.05], fear conditioning [F (1, 79) = 0.115, p>0.05], and the interaction between treatment x fear conditioning [F (3, 79) = 0.441, p>0.05] on walking duration (Figure 4.13A).

There were no significant effects of treatment [F (3, 79) = 1.354, p>0.05], fear conditioning [F (1, 79) = 2.532, p>0.05], and the interaction between treatment x fear conditioning [F (3, 79) = 1.674, p>0.05] on distance moved (Figure 4.13B).

Kruskal Wallis test revealed a significant difference among groups [χ^2 (7) = 19.173, p<0.01] in rearing duration. However, *post hoc* analysis with Dunn's test did not show any significant differences between groups (Figure 4.13C).

Two-way ANOVA revealed a significant effect of fear conditioning [F (3, 79) = 5.024, p<0.05], but not of treatment [F (1, 79) = 1.048, p>0.05] or the interaction between treatment x fear conditioning [F (3, 79) = 0.328, p>0.05] on grooming duration (Figure 4.13D). However, *post hoc* analysis with Student Newman-Keuls did not show any significant difference between groups (Figure 4.12D).

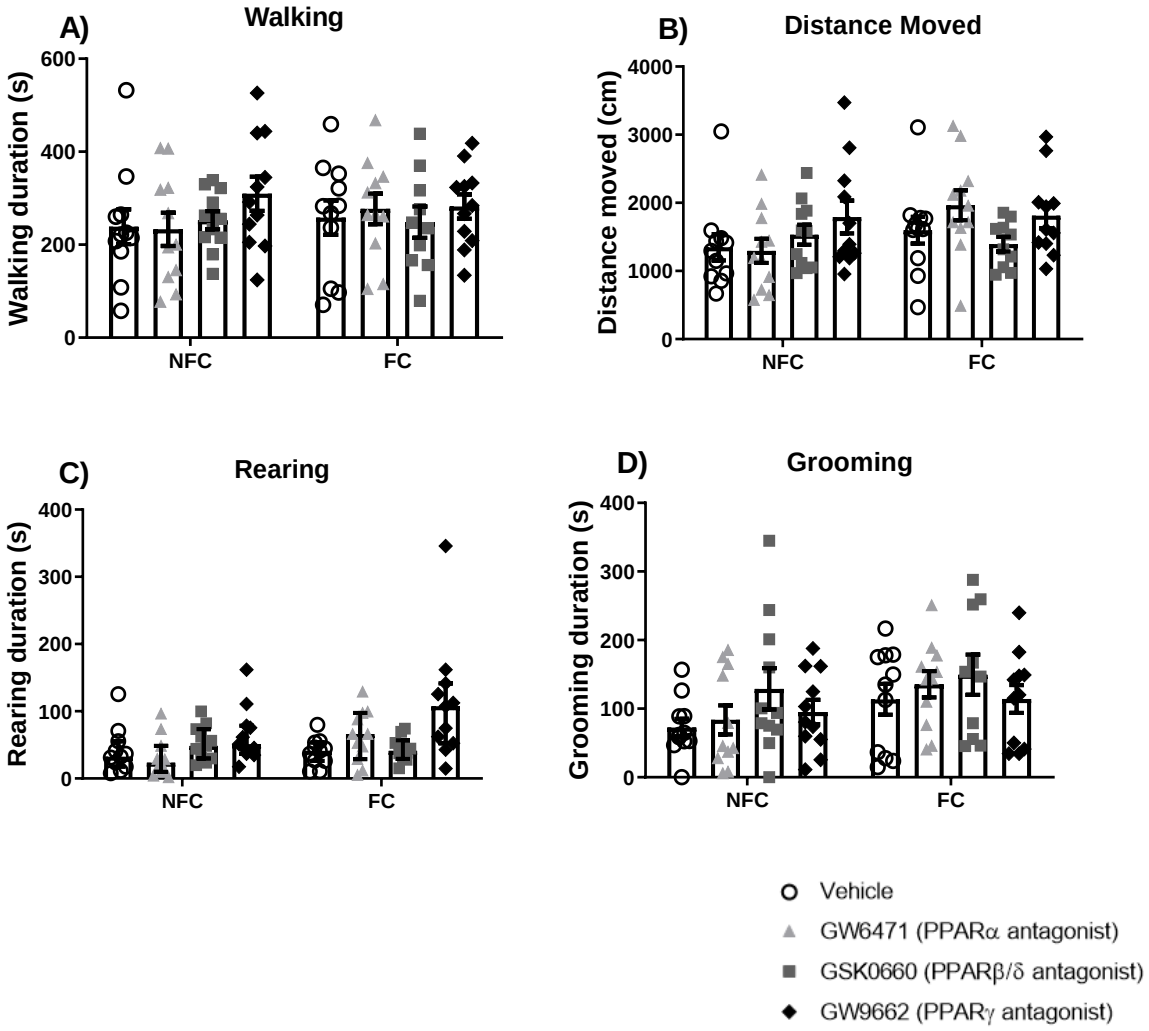


Figure 4.13: Effects of fear-conditioning and intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on walking duration (A), distance moved (B), grooming duration (C), and rearing duration (D). Data are expressed as mean ± S.E.M (A, B, D) or median with interquartile range and min/max (C) (n=7-10 rats per group).

4.3.3 Expression of PPARs in the CeA

4.3.3.1 Western Blotting

PPARα, PPARβ/δ and PPARγ expression was confirmed in the right and left CeA of naïve male SD rats (Figure 4.14). The bands for PPARα (55kDa) and PPARβ/δ (52kDa) were obtained with the use of a monoclonal antibody. As mentioned in the section 4.2.9.1, the double bands for PPARγ are a consequence of the expression of two subtypes of PPARγ: PPARγ$_1$ and PPARγ$_2$. The 42kDa band corresponds to β-actin, used as an endogenous control.

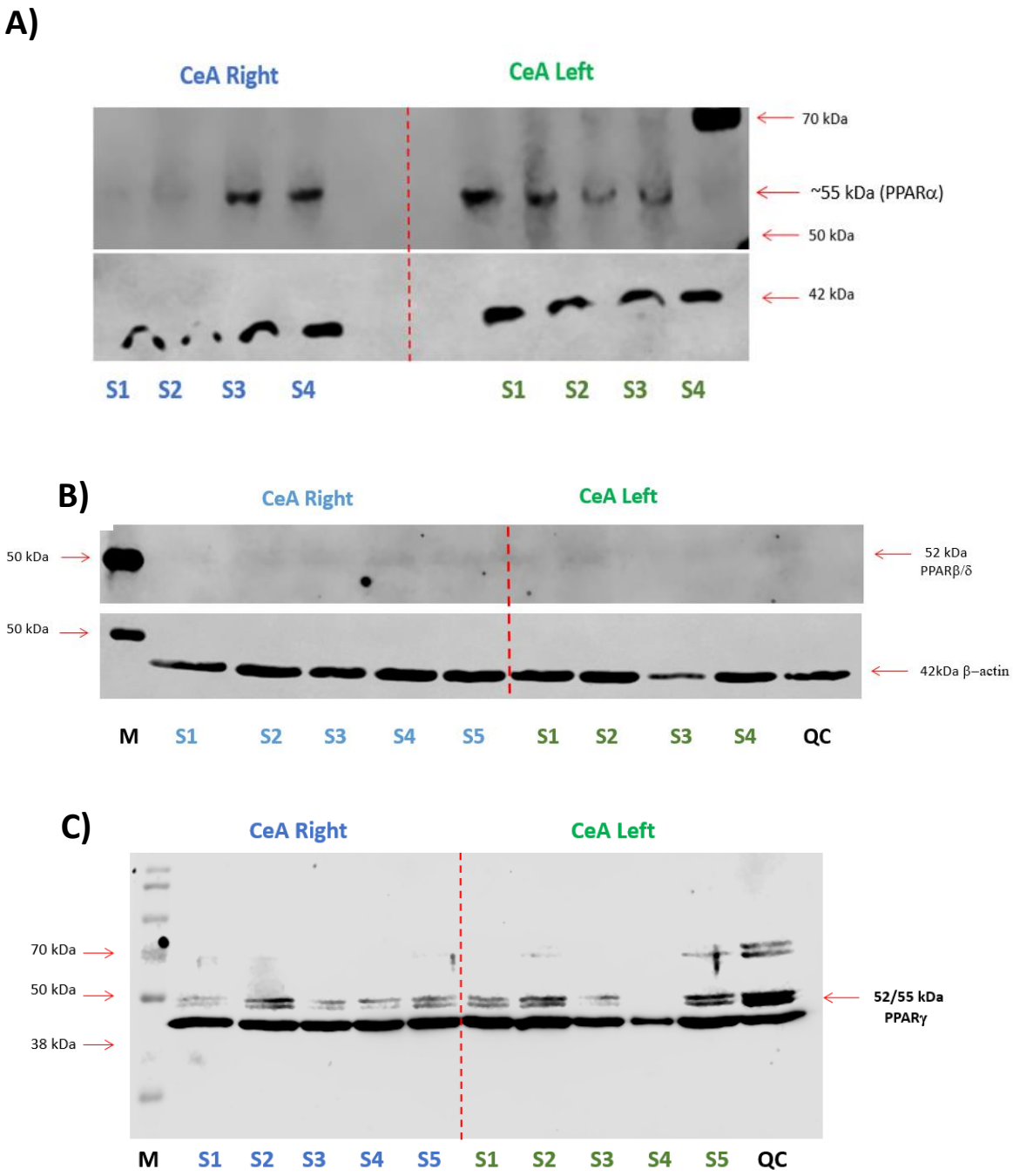


Figure 4.14: Expression of PPARα (A), PPARβ/δ (B), and PPARγ (C) in the right and left CeA (n=4-5 per side). The expression of PPARα is seen at 55kDa, PPARβ/δ at 52kDa, and PPARγ at 52/55kDa. β-actin was used as endogenous control. M=marker/ladder; QC=quality control.

4.3.3.2 RT-qPCR

The available antibodies developed to bind to PPARβ/δ in western blotting protocols did not give results that were entirely satisfactory, as evidenced by the faint bands above (Figure 4.14B). Therefore, we opted to demonstrate the presence of PPARβ/δ in the CeA using RT-qPCR. The presence of mRNA encoding PPARβ/δ was confirmed in the right and left BLA of naïve male SD rats. (Figure 4.15).

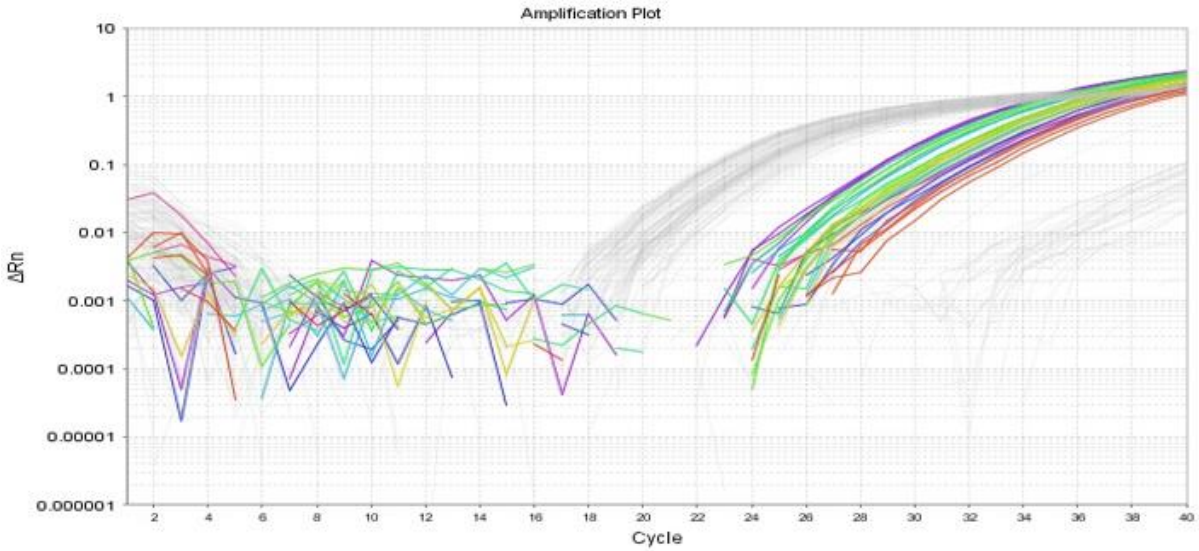


Figure 4.15: Amplification plots for PPARβ/δ gene expression in the CeA.

4.3.4 Effect of fear conditioning and intraplantar formalin or saline injections on the tissue levels of neurotransmitter, endocannabinoids and NAEs in the central nucleus of the amygdala (CeA)

It was observed that the duration of freezing in control, intra-CeA vehicle-injected rats was significantly higher in the experiments described in this chapter compared with those observed in the intra-BLA experiments described in Chapter 3. In order to investigate potential reasons for the differences in freezing levels between the two experiments described in this chapter (described in this section as intra CeA-Formalin and intra CeA-Saline) and in chapter 3 (described in this section as intra BLA-Formalin and intra BLA-Saline), I examined if the location of the cannula/microinjection (CeA or BLA) and the presence or absence of an inflammatory painful stimulus (Formalin or Saline) would affect the levels of neurotransmitters, endocannabinoids or NAEs that could result in differences

in behaviour. For that, we analysed punched CeA tissue from fear-conditioned, vehicle-treated rats from the four studies using LC-MS/MS.

4.3.4.1 Effect of fear conditioning and intraplantar formalin or saline injections on the levels of neurotransmitters in the central nucleus of the amygdala (CeA)

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 39.849, p<0.001] in GABA levels (Figure 4.16A). However, *post hoc* analysis with Dunn's test did not show any significant differences between groups. When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 15.628, p<0.05] but not in the left [χ^2 (7) = 4.291, p>0.05] side. However, *post hoc* analysis with Dunn's test did not show any significant differences between groups in GABA levels in the right CeA.

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 113.205, p<0.001] in glutamate levels (Figure 4.16B). *Post hoc* analysis with Dunn's test indicated lower levels of glutamate in the right (ipsilateral) CeA compared with left (contralateral) CeA in the following groups: NFC intra BLA-Saline, NFC intra CeA-Formalin, FC intra CeA-Formalin, and FC intra CeA-Saline (*p<0.05). When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 21.534, p<0.01] and in the left [χ^2 (7) = 34.424, p<0.001] side. *Post hoc* analysis with Dunn's test indicated higher levels of glutamate in the right CeA of NFC rats of the intra-CeA Saline group (NFC intra CeA-Formalin vs NFC intra CeA-Saline, $^{\$}$p<0.05). *Post hoc* analysis in the left CeA did not show any significant differences between groups.

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 101.676, p<0.001] in serotonin levels (Figure 4.16C). *Post hoc* analysis with Dunn's test indicated that FC animals with cannulae and microinjection into the BLA which received saline injections into the hind paw had higher levels of serotonin compared to the formalin-treated counterparts (Left FC intra BLA-Formalin vs Left FC intra BLA-saline, $^{\$}$p<0.05) in the left CeA. Likewise, NFC animals with cannulation/microinjection into the CeA which received saline injections into the hind paw had higher levels of serotonin compared to the formalin-treated counterparts (Right NFC intra CeA-Saline vs Right NFC intra CeA-Formalin, $^{\$}$p<0.05) in the right CeA. *Post hoc* analysis also indicated that NFC animals which received intraplantar saline injections into the hind paw with CeA cannulation/microinjection had

higher levels of serotonin compared to BLA cannulation/microinjection counterparts (Right NFC intra CeA-Saline vs Right NFC intra-BLA Saline, $^{\#}p<0.05$). When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 47.503, $p<0.001$] and in the left [χ^2 (7) = 50.757, $p<0.001$] CeA. *Post hoc* analysis with Dunn's test confirmed the changes in the right side described above. *Post hoc* analysis in the left CeA indicated that NFC animals with intra-BLA cannulation/microinjection which received intraplantar injection of saline had higher levels of serotonin compared to their formalin-injected counterparts (Left NFC intra BLA-Formalin vs Left intra BLA-Saline, $^{\$}p<0.05$).

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 57.275, $p<0.001$] in dopamine levels (Figure 4.16D). *Post hoc* analysis with Dunn's test indicated that NFC animals which received saline injections into the hind paw with cannulation/microinjection into the CeA had higher levels of dopamine compared to animals with counterparts that had cannulation/microinjection into the BLA (Right NFC intra CeA-Saline vs Right NFC intra BLA-Saline, $^{\#}p<0.05$) in the right CeA. When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 32.377, $p<0.001$] and in the left [χ^2 (7) = 23.094, $p<0.01$] CeA. *Post hoc* analysis with Dunn's confirmed the changes in the right side described above. *Post hoc* analysis in the left CeA did not indicate further significant differences between groups.

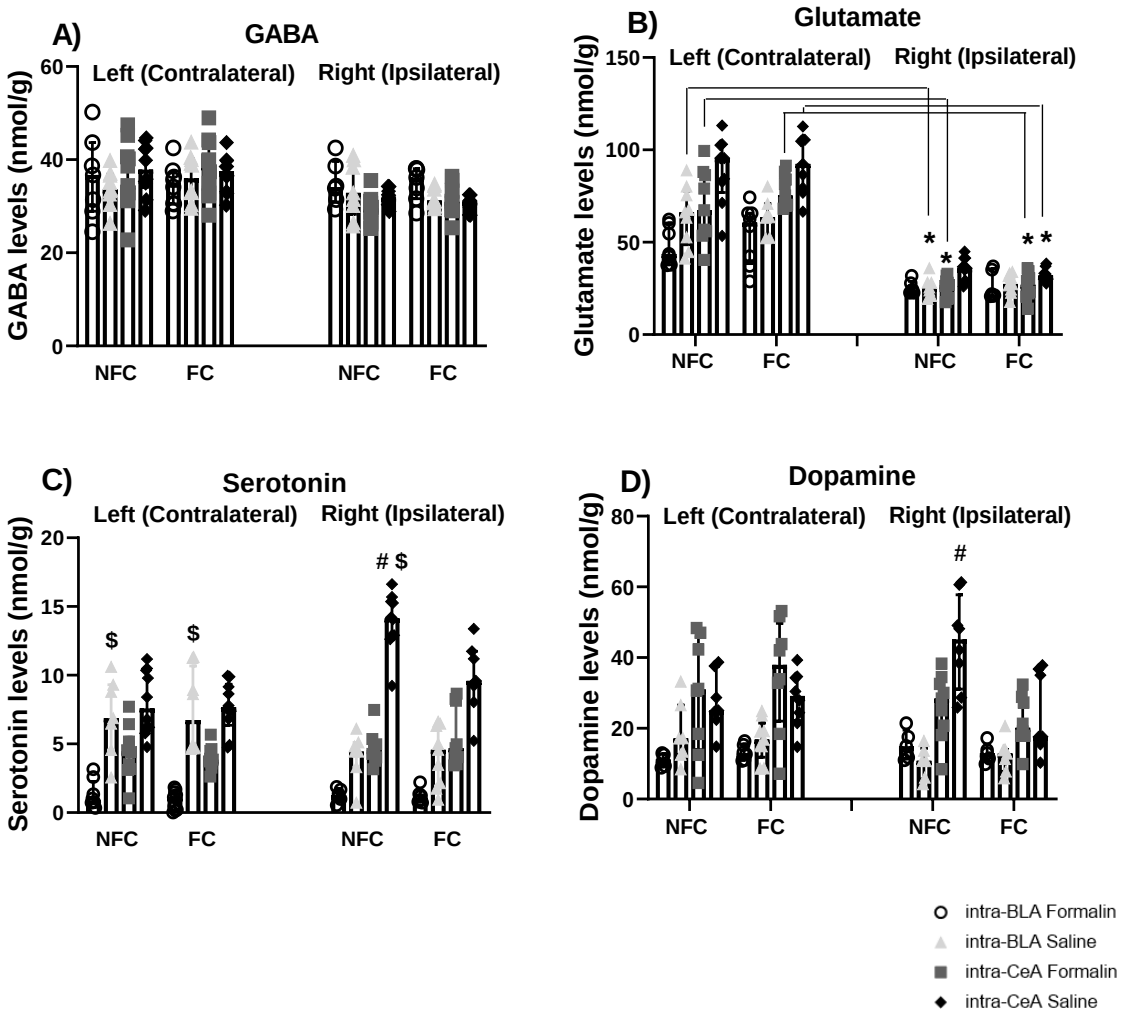


Figure 4.16: Effects of fear conditioning and intra-BLA or intra-CeA cannulation of selective PPARα, PPARβ/δ and PPARγ antagonists on the levels of neurotransmitters. Post hoc analysis indicated side effects on glutamate levels (*$p<0.05$, vs Left). FC and NFC intra-BLA Saline study rats had higher levels of serotonin compared to formalin-treated counterparts ($^{\$}p<0.05$ vs Formalin-treated counterpart) in the left CeA. NFC intra-CeA Saline rats had higher levels of serotonin compared to formalin-treated counterparts ($^{\$}p<0.05$) and to BLA counterparts ($^{\#}p<0.05$ vs BLA-Saline) in the right CeA. NFC intra-CeA saline rats had higher levels of dopamine compared to BLA counterparts ($^{\#}p<0.05$) in the right CeA. NFC intra-CeA Saline group had higher levels of glutamate in the right CeA (NFC intra CeA-Formalin vs NFC intra CeA-Saline, $^{\$}p<0.05$). Data are expressed as median with interquartile range and min/max (n=7-9 rats per group).

4.3.4.2 Effect of intraplantar formalin or saline injections on the levels of endocannabinoids and NAEs in the central nucleus of the amygdala (CeA)

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 73.306, p<0.001] in 2-AG levels (Figure 4.17A). *Post hoc* analysis with Dunn's test indicated that NFC animals which receive saline injections in the hind paw with cannulation into the CeA had higher levels of 2-AG compared to the animals with cannula implantation into the BLA (Right NFC intra CeA-Saline vs Right NFC intra BLA-Saline, $^{\#}$p<0.05) in the right CeA. *Post hoc* analysis also revealed that NFC animals with cannulation into the CeA which receive saline injections in the hind paw had higher levels of 2-AG compared to the formalin-treated counterparts (Right NFC intra CeA-Formalin vs Right NFC intra CeA-Saline, $^{\$}$p<0.05) in the right CeA. When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 30.831, p<0.001] and in the left [χ^2 (7) = 30.536, p<0.001] CeA. *Post hoc* analysis with Dunn's confirmed the changes in the right side described above. *Post hoc* analysis in the left CeA did not indicate further significant differences between groups.

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 87.431, p<0.001] in AEA levels (Figure 4.17B). *Post hoc* analysis with Dunn's test indicated side effect on the NFC intra CeA- Saline and FC intra CeA-Saline (Right vs Left *p<0.05). When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 19.138, p<0.01] and in the left [χ^2 (7) = 27.495, p<0.001] CeA. *Post hoc* analysis with Dunn's indicated that NFC and FC rats which received an intraplantar injection of formalin and had cannulae implanted into the CeA had lower levels of AEA compared to their counterparts with cannulae implanted into the BLA (Left NFC intra CeA-Formalin vs intra BLA-Formalin, $^{\#}$p<0.05; Left FC intra CeA-Formalin vs Left FC intra BLA-Formalin, $^{\#}$p<0.05). *Post hoc* analysis in the right CeA did not indicate further significant differences between groups.

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 106.074, p<0.001] in PEA levels (Figure 4.17C). *Post hoc* analysis with Dunn's test indicated side effect on the NFC and FC intra CeA-Saline and FC intra BLA-Saline (Right vs Left *p<0.05). When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 20.507, p<0.01] but not in the left [χ^2 (7) = 8.263, p>0.05] CeA. *Post hoc* analysis in the right CeA did not indicate significant differences between groups.

Kruskal Wallis test revealed a significant difference among groups [χ^2 (15) = 100.506, p<0.001] in OEA levels (Figure 4.17D). *Post hoc* analysis with Dunn's test indicated side effect on the NFC and FC intra CeA-Saline and FC intra CeA-Formalin (Right vs Left *p<0.05). When each side was analysed separately, Kruskal Wallis revealed a significant difference among groups in the right [χ^2 (7) = 15.668, p<0.05] but not in the left [χ^2 (7) = 10.940, p>0.05] CeA. *Post hoc* analysis in the right CeA did not indicate significant differences between groups.

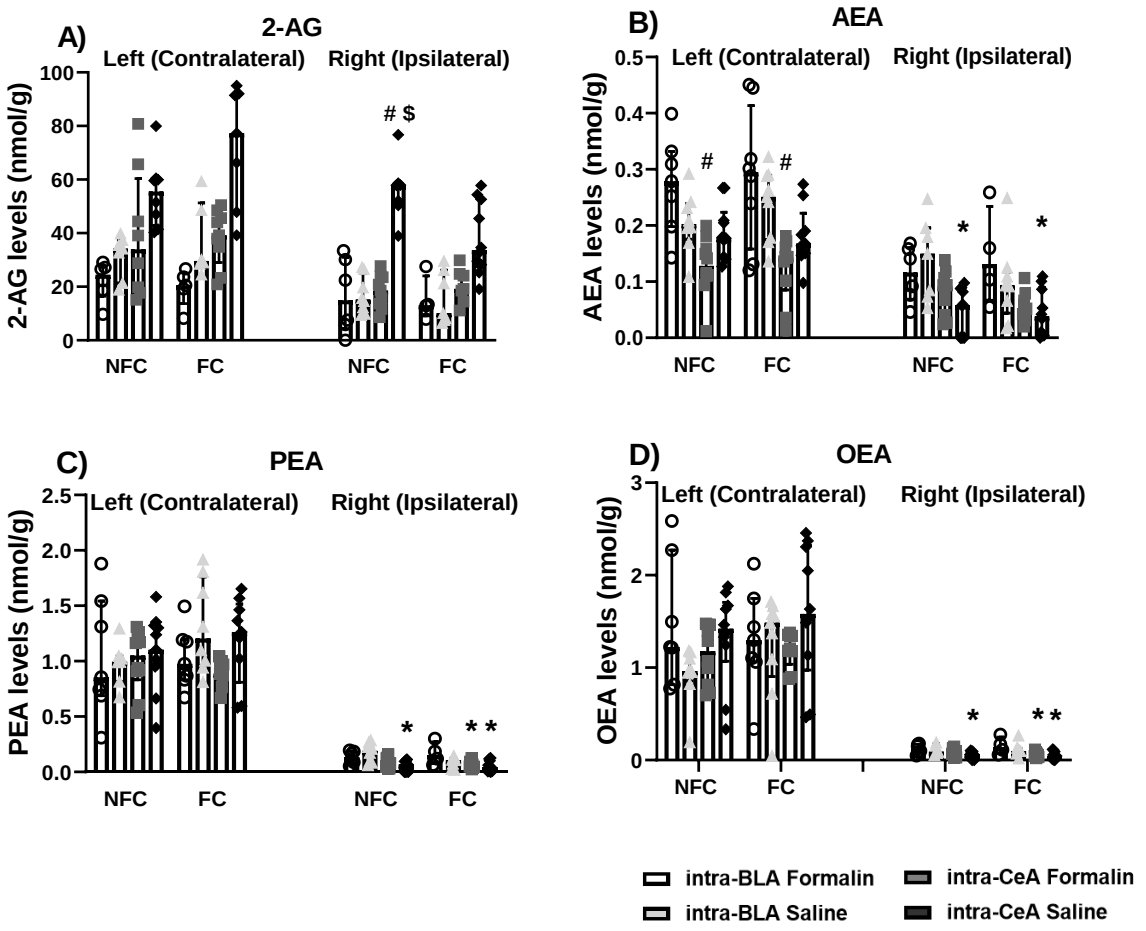


Figure 4.17: Effects of fear conditioning and intra-CeA administration of selective PPARα, PPARβ/δ and PPARγ antagonists on the levels of endocannabinoids and NAEs. Post hoc analysis indicated side effects on AEA, PEA and OEA levels (Right vs Left *p<0.05). NFC animals of the intra CeA-Saline study had higher levels of 2-AG compared to the intra-BLA counterparts ($^{\#}$p<0.05) and to the formalin-treated counterparts ($^{\$}$p<0.05) in the right CeA. NFC and FC rats of the intra-CeA Formalin study had lower levels of AEA compared to BLA counterparts ($^{\#}$p<0.05). Data are expressed as median with interquartile range and min/max (n=6-9 rats per group).

4.4 Discussion

The two experiments described in this chapter investigated a possible role of PPARs expressed in the CeA in the mediation or modulation of inflammatory pain, FCA, and conditioned fear in the presence versus absence of nociceptive tone. The expression of the three isoforms in the CeA was confirmed by western blotting and, for PPARβ/δ, RT-qPCR. Intraplantar administration of formalin evoked robust nociceptive behaviour, as demonstrated by CPS in the first experiment, while rats that received an intraplantar injection of saline (i.e. second experiment) had significantly lower/negligible CPS. Notably, fear conditioning resulted in only a very small reduction in formalin-induced nociceptive behaviour i.e. FCA, with no significant FCA observed in vehicle-treated rats. The administration of GW6471 (PPARα antagonist), GSK0660 (PPARβ/δ antagonist) and GW9662 (PPARγ antagonist) did not affect pain-related responses in NFC or FC rats. These results suggest that PPARs in the CeA do not mediate or modulate formalin-evoked nociceptive behaviour. As mentioned before, PPAR agonists and endogenous ligands have been shown to modulate pain responses (see Okine et al., 2018 for a review). To my knowledge, this is the first study to investigate the effects of the antagonism of PPARs expressed in the CeA on pain behaviour.

The results suggest that PPAR signalling in the CeA does not mediate or modulate formalin-evoked nociceptive behaviour. As previously discussed, several studies have demonstrated effects of PPAR agonists on pain-related behaviour (Okine et al, 2018) but less is known about the effect of PPAR antagonists. To our knowledge, the study described in the present chapter is the first study to investigate the effect of the blockade of PPARα, PPARβ/δ and PPARγ expressed in the CeA on inflammatory pain. Similarly to what was shown by Donvito et al. (2017) and Mansouri et al. (2017) in their systemic studies, by me in my own systemic studies described in Chapter 2 and in the intra-BLA study described in chapter 3, PPAR antagonism in the CeA do not affect formalin-evoked nociceptive behaviour. Therefore, PPARs expressed in the CeA do not seem to modulate inflammatory pain behaviour.

FCA was previously shown to be associated with increased levels of endogenous ligands of PPARs (Rea et al., 2013c) in the BLA, but less is known about PPAR signalling within the CeA in FCA. The results demonstrate that fear conditioning mildly reduced formalin-evoked

nociceptive behaviour, in contrast to the robust FCA observed in other studies (Roche et al., 2010; Ford et al., 2011; Rea et al., 2011, 2014; Butler et al., 2012; Olango et al., 2012) and those described in chapter 2 and 3. Lesions of the CeA was shown to abolish FCA (Helmstetter and Bellgowan, 1993). Therefore, the surgical procedure may have caused partial lesions of the CeA, which could have affected the expression of the FCA and explain the minor suppression of pain by conditioned fear seen in this study.. However, because the expression of FCA in this experiment was weak, the effects of PPAR antagonism on FCA in this study would have been difficult to determine.

It should also be noted that freezing duration was substantially higher in the NFC vehicle group of the second experiment (i.e. NFC intra-CeA-Saline) compared to the equivalent intra-BLA group described in Chapter 3 (i.e. NFC intra BLA-Saline). DMSO, the solvent chosen for the dilution of the drugs in these experiments, although widely used in neuroscience research and shown previously within our laboratory not to have a significant effect on formalin-evoked nociceptive behaviour when injected into the mPFC (Dr. Bright Okine, personal communication), can have bioactivity. For instance, a study from Lu and Mattson (2001) demonstrated that DMSO administration inhibited glutamatergic responses in hippocampal neurons. More recently, Penazzi et al. (2017) have investigated several effects of DMSO administration on brain structure and functioning and found an enhancement in hippocampal-dependent spatial memory accuracy, anxiolytic effects and increased spine density. Likewise, Zhang et al. (2017) also reported changes in neuronal morphology in *in vitro* studies in which primary cultured neurons and astrocytes were exposed to different DMSO concentrations. Therefore, it is possible that the administration of DMSO in the CeA may have influenced freezing behaviour and FCA expression. Optogenetic activation of the medial central amygdala (CEm) was shown to provoke unconditioned freezing (Ciocchi et al., 2010b). Moreover, muscimol inactivation of the lateral central amygdala (CEl) also resulted in increased unconditioned freezing, probably by inactivating a GABAergic inhibition of the CEm activity (Ciocchi et al., 2010b). With the use of optogenetics and tracing tools, it was demonstrated that PKC-δ^+ CEl neurons synapse onto CEm neurons which project to the PAG, a region which has been extensively reported to be involved in generating freezing (Haubensak et al., 2010). Therefore, I suggest that DMSO may be either directly activating CEm cellular activity leading to increased freezing or indirectly inactivating GABAergic inhibition from the CEl, which in turn results in increased freezing duration.

Fear and anxiety have very similar neurocircuitry and neurophysiological basis (Charney et al., 1998; Hofmann et al., 2012). Recent studies have pointed to a possible role of PPARs in anxiety. Youssef et al. (2019) have shown the administration of a PPARγ antagonist blocked the anxiolytic effect of beta-caryophyllene whereas another study demonstrated that repeated stress decreased PPARγ expression in the amygdala, and treatment with anxiolytics recovered PPARγ expression (Liu et al., 2018). Furthermore, PPARγ blockade or knockout was shown to have anxiogenic effects on mice (Domi et al., 2016). In this same study, intra-amygdala injections of pioglitazone (PPARγ agonist) were shown to reduce stress-induced anxiety behaviour in rats. In the experiments described in this chapter, the re-exposure to a context previously paired with footshock significantly increased freezing duration and defecation in both studies. However, the blockade of PPARs in the CeA did not affect context-induced freezing either in the presence or in the absence of nociceptive tone. These results indicate that PPARs in the CeA do not modulate contextually induced fear responses, and that the modulatory role of PPAR signalling is limited to the BLA. To my knowledge, this is the first experiment to investigate the effects of PPAR blockade in the CeA on fear responses.

The higher levels of freezing observed in the vehicle-treated group of the intra CeA-saline study were associated with increased tissue levels of dopamine and serotonin in the ipsilateral CeA. Serotonin and dopamine have both been linked to conditioned and unconditioned fear responses. The dopaminergic receptor D_2 has been shown to be expressed in the amygdala, with higher levels in the CeA (de la Mora et al., 2012). The VTA is a well-known source of dopaminergic neurons and projects to the amygdala through the mesolimbic pathway (Brandão and Coimbra, 2018). Although several studies have shown the importance of dopamine modulation in the BLA in conditioned fear responses (Guarraci et al., 2000; Greba et al., 2001; de Oliveira et al., 2011b; de Souza Caetano et al., 2013), less is known for unconditioned fear. Macedo et al. (2007) have shown that D_2 receptor-mediated signalling in the BLA is involved in the low expression of unconditioned freezing triggered by chemical stimulation of the inferior colliculus. The authors propose that the effects of dopaminergic transmission on defensive behaviour may depend on the type of emotional stimulus presented. Very little is known about the role of the dopaminergic system in the CeA in anxiety. De la Mora et al. (2012) revealed that infusions of low doses of a D_2 antagonist in the CeA increased burying behaviour, which is interpreted as anxiogenic. This result, together with the information coming from studies of conditioned behavioural

responses, suggest that blocking the activity of the dopaminergic system within the CeA has anxiogenic effects. The increased expression of freezing observed in the vehicle-treated group of the intra CeA-saline study were associated with increased tissue levels of serotonin in the ipsilateral CeA. Isosaka et al. (2015) have shown that intraperitoneal injection of serotonin antagonist increased innate fear-induced freezing expression and enhanced cellular activity in the CeA. In subsequent experiments, they demonstrated that pharmacological, pharmacogenetic and optogenetic suppression of serotonin signalling in the CeA increased the innate-freezing response. Therefore, the suppression of serotoninergic signalling within the CeA results in higher freezing levels. In my experiment, I observed greater levels of dopamine and serotonin in the group which elicited high levels of unconditioned freezing (i.e. NFC intra CeA-vehicle treated rats, compared to the NFC vehicle-treated group of the other studies). I speculate that these higher levels of dopamine and serotonin are triggered by DMSO effects on the neurophysiology of the amygdala, which in turn results in higher unconditioned responses. Specifically, DMSO within the CeA may lead to diminish receptor binding of dopamine and serotonin, causing their increased extracellular levels, and resulting in higher freezing duration even in the absence of an aversive stimulus or previous association to an aversive stimulus in the absence of nociceptive tone. Further work would however be required to test this hypothesis. The enhancement in freezing expression observed in the vehicle-treated group of the intra CeA-saline study was also associated with increased tissue levels of glutamate in the ipsilateral CeA. Although the glutamatergic system in the BLA has been the subject of several studies, the role of glutamate in the CeA in fear expression was less explored. One study has shown that microinjection of a glutamate antagonist (i.e. MPEP) into the CeA had anxiolytic effects (De La Mora et al., 2006) and disrupted fear learning (Walker and Davis, 2002b). Therefore, I hypothesize that microinjections of DMSO into the CeA led to an augmentation of glutamate levels that resulted in increased freezing duration in the absence of a nociceptive tone. Further studies would be necessary to investigate this hypothesis.

In conclusion, these results indicate that PPARs expressed in the CeA do not modulate pain or pain-fear interaction responses. Additionally, the blockade of PPARs in the CeA did not alter freezing expression. Thus, the effects of PPAR blockade in fear expression seen in the previous chapter seem to be exclusive to the BLA subnucleus. Interestingly, in the present chapter, I have observed possible effects of the microinjection of DMSO into the CeA on

freezing expression in the absence of nociceptive tone, but further investigations are necessary to elucidate these results.

The experiments described in chapter 2, 3 and 4 have indicated that PPARs may modulate short-term fear extinction and anxiety. Therefore, it remained to be explored whether these receptors play a role in innate anxiety responses and cognitive performance, and whether this differs in the presence versus absence of pain. This question will be addressed in chapter 5.

Chapter 5. Effects of systemic administration of PPARα, PPARβ/δ and PPARγ antagonists and PEA, an agonist at PPARs, on innate anxiety and cognition in the presence and absence of chronic inflammatory pain.

5.1 Introduction

In chapters 3, I have demonstrated that the blockade of PPARα and PPARγ in the BLA prolongs freezing duration in FC rats in the presence of a noxious stimulus (i.e. intra-plantar injection of formalin). It is possible that these effects may be associated with PPAR modulation of memory formation or recall. Additionally, the data also indicated that the blockade of the three isoforms of PPARs in the BLA increases freezing duration in NFC rats in the absence of formalin-evoked nociceptive tone. Those data suggest that the PPAR signalling system may also be involved in innate (in addition to conditioned) anxiety responses. Therefore, in the present chapter, I investigate if PPAR signalling has a modulatory effect on anxiety and cognitive responses. In addition, because the previous studies described have shown different outcomes of the blockade of PPARs on conditioned fear responses depending on the absence or presence of pain, I examined if the presence of chronic inflammatory pain affects PPAR-mediated modulation of anxiety and cognitive responses.

All three subtypes of PPARs are expressed in brain regions that play key roles in cognition and anxiety (Moreno et al., 2004; Warden et al., 2016) such as the amygdala, PFC and hippocampus. However, few studies have investigated the role of PPARs in anxiety and cognition. There is some evidence that PPARs modulate anxiety. Endogenous ligands at PPARs have been shown to be increased in response to stress or anxiety (Bluett et al., 2014; Hillard, 2018). A recent clinical study has also shown that the levels of OEA are significantly lower in PTSD patients compared to controls (Wilker et al., 2016). Additionally, administration of PEA attenuated aggressiveness in a social isolation model of PTSD in mice (Locci et al., 2017). Fernandez et al. (2009) revealed that naringin, a bioflavonoid isolated from citrus fruits which is an endogenous ligand of PPARγ, had anxiolytic and antidepressant effects. Another study indicated that seipin knockout (Seipin-KO) male mice displayed anxiety- and depression-like behaviour, associated with decreased levels of mRNA and protein levels of PPARγ in the hippocampus and cortex (Zhou et al., 2014) and the administration of rosiglitazone attenuated the anxiety-like behaviour in male Seipin-KO

mice. PPARγ genetic deletion had anxiogenic effects in mice (Domi et al., 2016). In this same investigation, the authors showed that systemic and intra-amygdalar injections of pioglitazone (PPARγ agonist) reduced stress-induced anxiety behaviour in rats, and that these effects were blocked by the administration of the PPARγ antagonist GW9662. Importantly, the systemic administration of GW9662 alone did not alter anxiety-related behaviour. Rosiglitazone elicited antidepressant and anxiolytic-like behavioural effects in wild-type mice and pre-treatment with the PPARγ selective antagonist GW9662 blocked the effects of rosiglitazone (Guo et al., 2017). Recently, administration of pioglitazone was shown to attenuate harmaline-induced anxiety-like behaviours and spatial learning and memory impairments (Aghaei et al., 2019), similar to what was observed with rosiglitazone-treated animals. Likewise, Youssef et al. (2019) have shown that the administration of GW9662 blocked the anxiolytic effect of beta-caryophyllene. A different study demonstrated that repeated stress decreased PPARγ expression in the amygdala, and treatment with anxiolytics recovered PPARγ expression (Liu et al., 2018). One study also investigated the role of PPARγ in fear responses. Gemma et al. (2004) demonstrated that young and aged rats fed with a diet rich in rosiglitazone had increased freezing duration in a context-induced fear protocol. In addition, the levels of PEA where shown to be increased in the BLA of FC rats (Rea et al., 2013a).

Various studies have investigated the effects of FAAH inhibitors on mnemonic tasks, and the effects of elevated FAAH substrate levels, particularly AEA, are often mediated by CB_1 receptors. Nevertheless, administration or manipulation of the levels of endogenous ligands at PPARs, some of which are FAAH substrates, have also been shown to enhance cognitive performance (Campolongo et al., 2009a; Goonawardena et al., 2011; Morena et al., 2014; Kramar et al., 2017; Rueda-Orozco et al., 2017; Scuderi et al., 2018; Segev et al., 2018; Zimmermann et al., 2018; Boccella et al., 2019). A few studies indicate a possible modulatory effect of PPARs on memory and learning processes. Mazzola et al (2009) have shown that the administration of URB597 (FAAH inhibitor) enhanced the learning of a passive avoidance test and this enhancement was attenuated by the administration of a PPARα antagonist, MK886. Following this result, these authors also demonstrated that the administration of a PPARα agonist, WY14643, produced similar effects to those observed with URB597, and these effects were also blocked by MK886. Also, a study from Campolongo et al. (2009) indicated that the administration of OEA improved learning of passive avoidance and spatial memory task when given immediately post-training and that

the actions of OEA were mimicked by the PPARα agonist GW7647 and are absent in PPARα null mice. Recently, Ratano et al. (2017) have shown that the cognitive enhancing effects of URB597 were dependent on PPARα, as well as CB_1 receptors and $TRPV_1$. Together, these studies indicate a modulatory role of PPAR signalling system in memory acquisition and consolidation.

PPARs modulate pain responses (for review see Okine et al 2018). Previous studies have shown that the selective activation of PPARα (LoVerme et al., 2006; Russo et al., 2007; Sagar et al., 2008; D'Agostino et al., 2009; Okine et al., 2014), PPARβ/δ (Gill et al., 2013a; Lyons et al., 2017), and PPARγ (Oliveira et al., 2007; Churi et al., 2008; Morgenweck et al., 2010; Hasegawa-Moriyama et al., 2012; Griggs et al., 2015; Mansouri et al., 2017b) has antinociceptive effects. The administration of PEA, an agonist at PPARs, also has antinociceptive effects in rodents (LoVerme et al., 2006; D'Agostino et al., 2007, 2009b; Costa et al., 2008; Sasso et al., 2012; de Novellis et al., 2012; Bettoni et al., 2013; Wang et al., 2014b; Di Cesare Mannelli et al., 2015; Donvito et al., 2015, 2016; Okine et al., 2016) and in humans (Keppel Hesselink and Hekker, 2012; Gabrielsson et al., 2016). Likewise, administration of the endogenous PPAR ligand OEA, and OEA-derived compounds, diminishes nociceptive behaviour (Vasconcelos et al., 2006; Suardíaz et al., 2007; Guida et al., 2015).

Pain can impact significantly on both anxiety (Scott et al., 2016) and cognition (Moriarty et al., 2011). Moreover, co-morbidity of chronic pain with anxiety disorders and/or cognitive impairment is very prevalent (de Heer et al., 2014b, 2018; Gerrits et al., 2015). PPAR isoforms are expressed in brain regions that are commonly implicated in pain, anxiety and cognition such as the amygdala (Warden et al., 2016), PFC (Moreno et al., 2004; Okine et al., 2014; Warden et al., 2016), hippocampus (Moreno et al., 2004; Domi et al., 2016) and PAG (Okine et al., 2017). As pointed out before, the previous results described in chapters 2 and 3 revealed a differential effect of pharmacological manipulation of PPARs on conditioned fear responding depending on the presence or absence of pain.

In the present chapter, I investigated the hypothesis that PPARs modulate innate anxiety responses and mnemonic function. Specifically, I examined the effects of intraperitoneal administration of GW6471 (PPARα antagonist), GSK0660 (PPARβ/δ antagonist), GW9662 (PPARγ antagonist), and PEA on the elevated plus maze (EPM), open field (OF), light-dark box (LDB), and novel object recognition (NOR) tests in rats in the presence and absence of

chronic inflammatory pain induced by intra-plantar injection of complete Freund's adjuvant (CFA). I also investigated the effects of systemic administration of the drugs on mechanical allodynia induced by CFA using the von Frey test. Therefore, the specific aims of the study described in this chapter were:

- To determine if PPAR signalling plays a role in innate anxiety responses by examining the effects of intraperitoneal administration of PPAR antagonists and PEA on behaviour of rats in the EPM, OF and LDB tests.
- To determine if PPAR signalling plays a role in cognitive performance by examining the effects of intraperitoneal administration of PPAR antagonists and PEA on behaviour of rats in the NOR test for recognition and spatial memory.
- To determine if PPAR signalling plays a role in mediating or modulating chronic inflammatory pain-related behaviour by examining the effects of intraperitoneal administration of PPAR antagonists and PEA on mechanical allodynia measured by von Frey testing in rats.
- To assess innate anxiety and recognition and spatial memory in the rat CFA model of chronic inflammatory pain.
- To investigate whether the presence or absence of nociceptive tone influences PPAR-mediated regulation of anxiety and cognitive responses.

5.2 Materials and Methods

5.2.1 Animals

Experiments were carried out on a total of 80 adult male Sprague-Dawley rats (230-250g on arrival; Envigo UK, Bicester, England). The animals were maintained at controlled temperature (22±2°C) and humidity (45-55%) under standard lighting conditions (12:12h light-dark cycle, lights on from 07.00h). Animals were housed 2-3 per flat bottomed cage (L:45 x H:20 x W:20cm) containing 3Rs paper bedding material (Fibrecycle Ltd., North Lincolnshire, United Kingdom) and sizzle nest material (LBS Biotechnology, Horley, United Kingdom) for the first week after arrival, and were posteriorly singly housed or the rest of the experiment. 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.

5.2.2 Drugs

GW6471, GSK0660, GW9662 and PEA (all obtained from Tocris Bioscience, Bristol, UK) were dissolved in a 1:1:8 (ethanol, cremophor; saline) vehicle solution. The doses of GW6471 (2 mg/kg), GSK0660 (1mg/kg) and GW9662 (2mg/kg) were chosen based on studies in the literature demonstrating their efficacy in reversing the antinociceptive and neuroprotective effects of PEA (Paterniti et al., 2013; Donvito et al., 2016) or pioglitazone (Mansouri et al., 2017; Griggs et al., 2015; Morgenweck et al., 2013), as well as the results presented in Chapter 2. Immunogenic complete Freund's adjuvant emulsifier (CFA, desiccated Mycobacterium tuberculosis in an 85% mineral oil, 15% mannide monooleate suspension, Sigma-Aldrich, Dublin, Ireland) was used to induce a chronic inflammatory pain state (Stein et al., 1988). Rats received a single 50 µl intraplantar injection of CFA (1 mg/ml) into the right hind paw, under brief isoflurane anaesthesia (3% in 0.8 L/min O_2). Control rats underwent intraplantar needle insertion to the right hind paw, also under isoflurane anaesthesia.

5.2.3 Experimental Design

The animals were kept in groups of three and allowed 4 days of habituation upon arrival. Five days after arrival, seven days before Complete Freud Adjuvant (CFA) injections, rats were singly housed. At day 7 and 10 after arrival (5 and 2 days before CFA injection), the baseline paw withdrawal thresholds in the von Frey test for mechanical allodynia were determined (Figure 5.1). The decision on taking two baseline values was based on previous

work in our group showing that response thresholds are lower at second baseline test session compared with the initial session. The von Frey test is detailed on section 5.2.4.1.5. Briefly, the rats were placed in one of the six chambers of the von Frey apparatus, where they were allowed to habituate for 15min. Then, the rats received 9 stimulations of each paw using the von Frey filaments according to the up-and-down method described by Dixon (Dixon, 1965). All responses were recorded and analysed afterwards. On day 12 after arrival, day 7 after single housing, the rats were divided into two groups: the rats allocated to the CFA-treated group received a 50µl intra-plantar injection of CFA into the right hind paw under isoflurane (2-3% in O_2, 0.8L/min) anaesthesia, while animals belonging to the No-CFA group had an equal-calibre needle inserted into the right hind paw also under isoflurane anaesthesia. After injections, the rats were immediately returned to their home cages. On the following day, the first post-CFA paw withdrawal threshold data collection took place, following the same protocol described in section 5.2.4.1.5, and another set of data was collected on day 7 post-CFA injection.

On day 21 post-CFA, the rats were tested for anxiety behaviour. The animals received an intraperitoneal injection of GW6471 (2mg/kg), GSK0660 (1mg/kg), GW9662 (2mg/kg), PEA (2mg/kg) or vehicle in an injection volume of 3ml/kg. Thirty minutes after injections, the rats underwent a series of anxiety tests: they were initially placed in the EPM arena (detailed on section 5.2.4.1.1) for 5min, followed by the OF test (detailed on section 5.2.4.1.2) for 5min, and then the LDB test (detailed on section 5.2.4.1.3) also for 5min. After all anxiety tests were completed, the rats were again placed in the von Frey apparatus for a 15min habituation followed by assessment of paw withdrawal thresholds. The rats were returned to their home cages after von Frey testing. On day 26 post-CFA injection, we initiated the NOR protocol described in detail in section 5.2.4.1.4. Briefly, on the first day of the protocol (day 26 post-CFA), rats were allowed to explore the NOR arena, that at this point had no objects, in a habituation trial for 10min. On the next day (day 27 post-CFA), the rats were exposed to the familiarization phase, in which they were allowed to explore freely for 5 minutes in the arena where three plastic bottles filled with water were now placed (according to specifications detailed below). This protocol was repeated 3 times, with 5-minute breaks between exposures. After the third exposure, the rat was returned to its home cage. On the test day (day 28 post-CFA), the animals received an intraperitoneal injection of GW6471 (2mg/kg), GSK0660 (1mg/kg), GW9662 (2mg/kg), PEA (2mg/kg) or vehicle in an injection volume of 3ml/kg. Rats were pseudorandomly re-assigned to drug treatment

groups relative to the treatments they received prior to anxiety testing on day 21 post-CFA using the Latin Square Randomisation method. Thirty minutes after administration of drugs, the rats were placed in the NOR arena for 5min, with one of the plastic bottles replaced by a squared plastic structure. The time spent exploring the familiar water-filled bottles and the novel object was recorded and later analysed. Again, when the NOR test was finished, the rats were placed in the von Frey apparatus for a 15min habituation followed by the sixth and final paw withdrawal threshold test. After the von Frey data collection, rats were euthanized by live decapitation, and the brain and spinal cord were harvested, snap frozen on dry ice, and stored at −80°C.

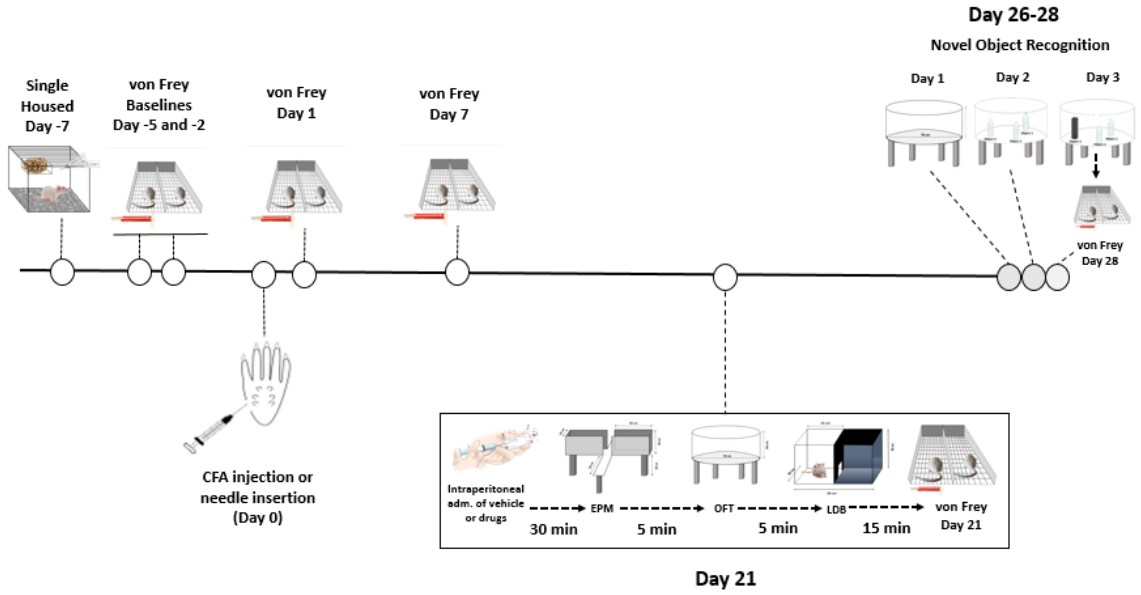


Figure 5.1: Graphical representation of the experimental design

5.2.4 Behavioural Tests

5.2.4.1 von Frey test for mechanical allodynia

The von Frey test apparatus comprised a six-chambered arena made of clear Perspex front and back walls and white chipboard lateral walls. The dimensions of the chambers were such that rats could move freely (14cm x 20cm x 25cm). A Perspex lid with air-holes was placed on top of the arena during the habituation and testing periods. In all experiments, the arena was placed on a raised wire-mesh flooring so that the experimenter could access the hind paws of the rats from below. Six rats were tested per session, and the arena was thoroughly cleaned between each session using 70% ethanol. Rats received an initial habituation period of 15min during which they were placed in individual chambers of the arena. No testing was

carried out, and at the end of the 15-minute period, the tests started. The baseline withdrawal thresholds were acquired twice, on days -5 and -2, before CFA intraplantar injection on day 0. For the statistical analysis, the second baseline was used. I applied the up-and-down method described by Dixon (Dixon, 1965). In this method, the rats receive a maximum of 9 nylon von Frey filament stimulations (Touch Test Sensory Evaluator #58011, Stoelting, Illinois, USA), starting with the 2g filament. Each filament was applied only once, perpendicular to the plantar surface of the hind paw, targeting the area at the base of the third and fourth digits (from medial to lateral) according to previous protocol used by the group, with sufficient force to cause slight buckling of the filament, for approximately 6 seconds or until a positive result was observed. A positive result was recorded if flinching, licking or withdrawal of the paw occurred on application of the filament or immediately after removal of the filament. Filaments were applied to both left and right hind paws (alternating between paws). First, the responses for the 2g filament for the contralateral paws of all six rats were collected; only then, the responses the 2g filament for the ipsilateral paws were collected. If a positive response was observed using the 2g filament for one or both of the paws, filaments of lower weights (down) were applied in descending order until no positive responses were observed in that paw. If there was no response using the 2g filament for one or both of the paws, filaments of higher weights (up) were applied in ascending order until a positive response was observed in that paw. A maximum of 9 stimuli were applied (see Appendix D for an example of the test table) in each paw. These nine digits generate a code that is associated to a constant (κ) detailed by Dixon (Appendix C). The final value for the threshold response is the result of the formula: $10^{[(\log (\text{last hair}) + \kappa)*0.3]}$.

5.2.4.2 Elevated plus Maze

The EPM arena consisted of a white wooden plus-shaped maze elevated 50cm from the room floor, with two arms enclosed by walls (30cm) and two open arms (Figure 5.2); the floor was covered in a black rubber material. Each arm was 50cm in length and 10cm in width and the arms were interconnected by a central platform. A video camera was positioned over the maze and the light levels were fixed at 60lux in the open arms and 25lux in the closed arms, according to the protocol previously used by our group. The rat behaviour was recorded and analysed using a computerized video tracking system (EthoVision® XT11.5, Noldus, the Netherlands) for a 5min period. The EPM was cleaned between animals with 70% Ethanol. Reduced time spent in the open arms(s) was used as an experimental

index of anxiety. Entries in arms were defined as entry of the rat's centre of gravity into the arms (centre point on the body).

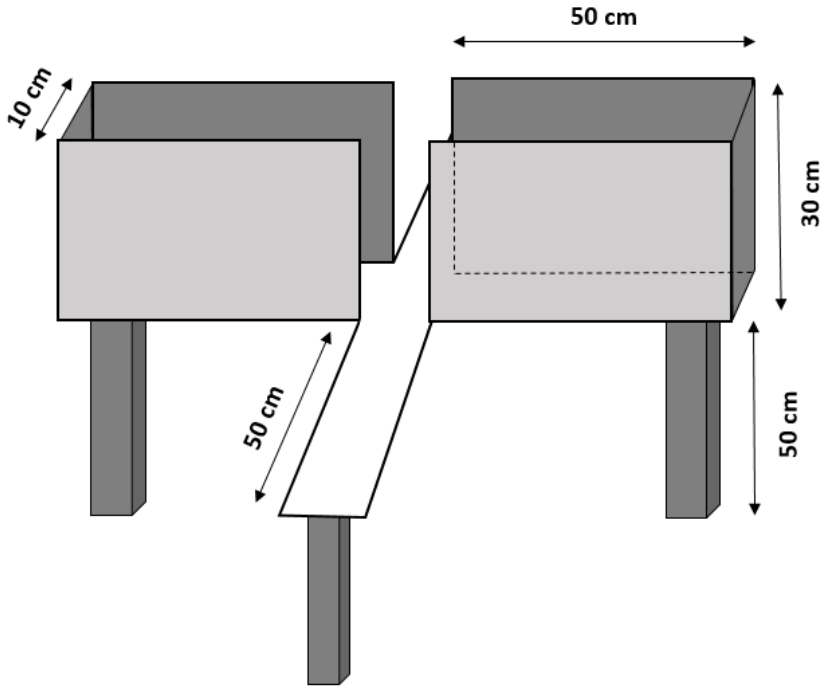


Figure 5.2: Graphical representation of the EPM arena.

5.2.4.3 Open Field Test

Behaviour in the open field was assessed once according to the experimental design described above (Figure 5.1). The rats were placed into a brightly lit (200lux) open field environment (diameter 75cm and 40cm high walls, of lective aluminium walls and floor; Figure 5.3). A camera positioned 35cm above the floor of the arena allowed for behaviour to be captured, recorded and assessed using a computerized video tracking system (EthoVision® XT11.5, Noldus, The Netherlands) for a 5min period. The open field was cleaned between animals with 70% ethanol. The behavioural assessment included locomotor activity (total distance moved) and duration of time spent (seconds) in the centre zone (45cm diameter). Reduced time spent in the centre zone is interpreted as anxiety-related behaviour.

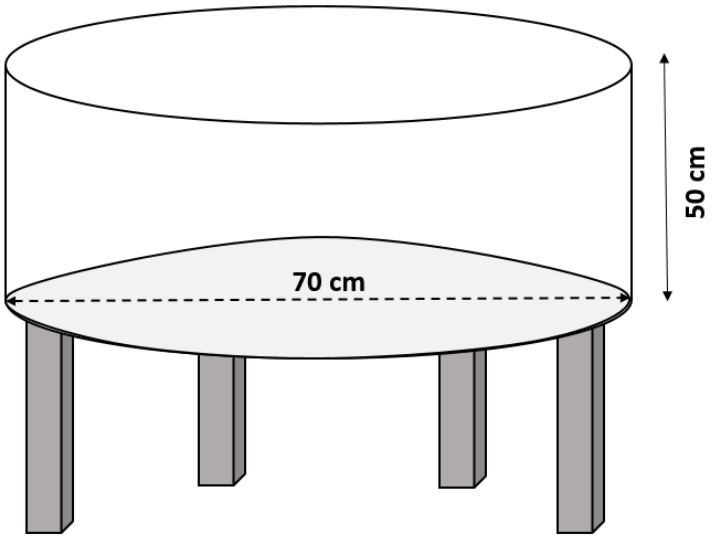


Figure 5.3: Graphical representation of the OF test arena.

5.2.4.4 Light-Dark box

Behaviour in the light-dark box was assessed once according to the experimental design described above (Figure 5.1). The rats were placed into a 30cm x 30cm x 30cm perplex chamber divided in two compartments that were connected by an entrance. One of the compartments is defined as light-chamber and was illuminated (150lux) while the other was called dark-chamber (0lux at the corners and 5lux next to the passage door (see Figure 5.4). A camera was positioned below the arena and allowed for behaviour to be captured, recorded and assessed using a computerized video tracking system (EthoVision® XT11.5, Noldus, The Netherlands) for a 5min period. The light-dark box arena was cleaned between animals with 70% ethanol. The behavioural assessment included locomotor activity (total distance moved), duration of time spent (seconds) in each of the chambers and number of entrance in the dark chamber. Reduced time spent in the light compartment is interpreted as anxiety-related behaviour.

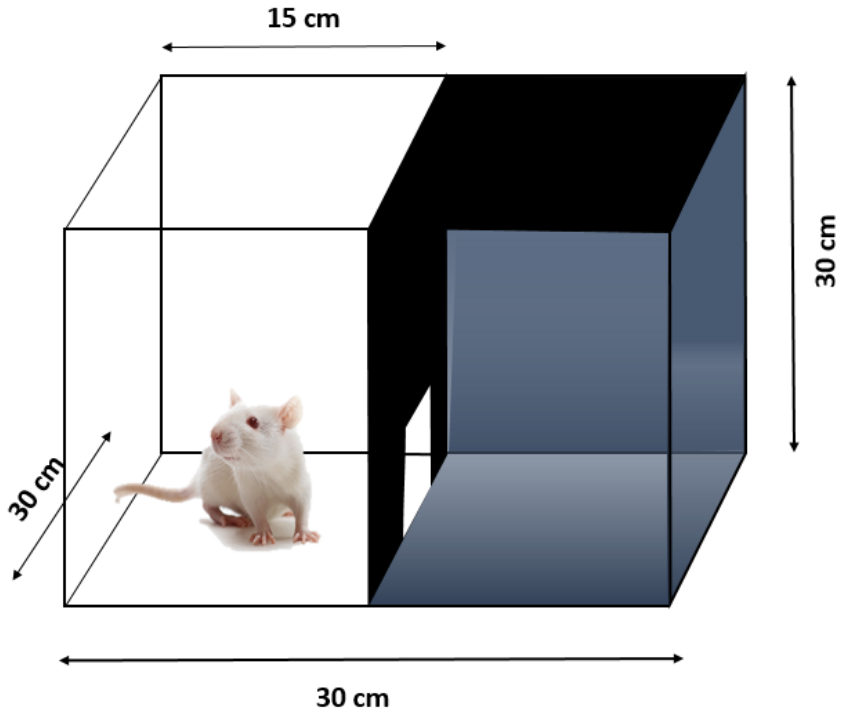


Figure 5.4: Graphical representation of the LDB arena.

5.2.4.5 Novel Object Recognition

Testing was carried out in in the same circular arena used for open field test (see Figure 5.3). In all experiments, the arena was illuminated by constant light intensity of 150±10lux at floor level of the arena. A camera positioned above recorded the whole test for subsequent analysis. The objects used included 500ml unlabelled transparent thin plastic polyethylene terephthalate Coca-Cola® bottles filled with water, and an abstract plastic structure with base 5cm × 5cm and height 16cm constructed from a mixture of green, white and blue toy blocks (Playskool Clipo® blocks). In all cases, the objects had no apparent natural significance to the rats and were secured to the base of the arena with white tack such that they were difficult to displace. Animals were habituated to the arena in the absence of objects for 10min on day 1 (see Figure 5.1). On the second day (familiarisation), three identical objects (Coca-Cola® bottles) were placed in the arena 16cm from points on the perimeter of the circular arena. The rat was allowed to freely explore the arena and objects three times for 5min, with 5min intervals between exposures. After this exposure, the animal was removed from the arena and returned to its home cage. On the following day (test), one of the objects was replaced with a novel object (abstract plastic structure constructed with a mixture of green, white and

blue toy blocks). The animal was allowed to freely explore the arena and objects for a period of 5min and then returned to its home cage. Representative images from the familiarisation exposure and test exposure are shown in Figure 5.5. The arena was cleaned with 70% ethanol and faecal pellets were removed between each exposure to remove odours and olfactory cues. Exploration of an object was defined as sniffing the object, rearing against the object or having the head directed towards the object within 2cm of the object. In all cases the experimenter assessing the behaviour was blind to the experimental treatment of the rat (CFA or drug). Ethovision XT11.5 was also used to track the distance (in cm) moved by the animal during testing. The position of the novel object was alternated between rats in order to minimise potential confounding effects related to orientation biases. Three indices were calculated in order to assess NOR results: (1) the preference ratio, defined as the time spent preferentially exploring the novel object in relation to the time spent exploring the familiar object in the same position, (2) the discrimination ratio, defined as the time spent exploring the novel object in relation to the time spent exploring the familiar objects in the test day, and (3) spatial discrimination ratio, defined as the time spent in the location of the new object in relation to the time spent in the same location in the familiarisation phase (the equations used in each of these rations can be seen in the table 5.1).

Index	Day	Equation
Preference index	Familiarisation Day	$T_{O3}/ (T_{O1}+T_{O2}/2) + T_{O3}$
	Test Day	$T_N/ (T_{O1}+T_{O2}/2) + T_N$
Discrimination index	Test Day	$T_N/ (T_{O1}+T_{O2}/2) + T_N$
Spatial Discrimination index	Familiarisation and Test Days	$T_N - T_{O3} / T_N + T_{O3}$

Table 5.1: Equations for the indices used in the assessment of NOR behaviour. T_{O1} = time exploring Object 1, T_{O2} = time exploring Object 2, T_{O3} = time exploring Object 3, and T_N = time exploring the new object.

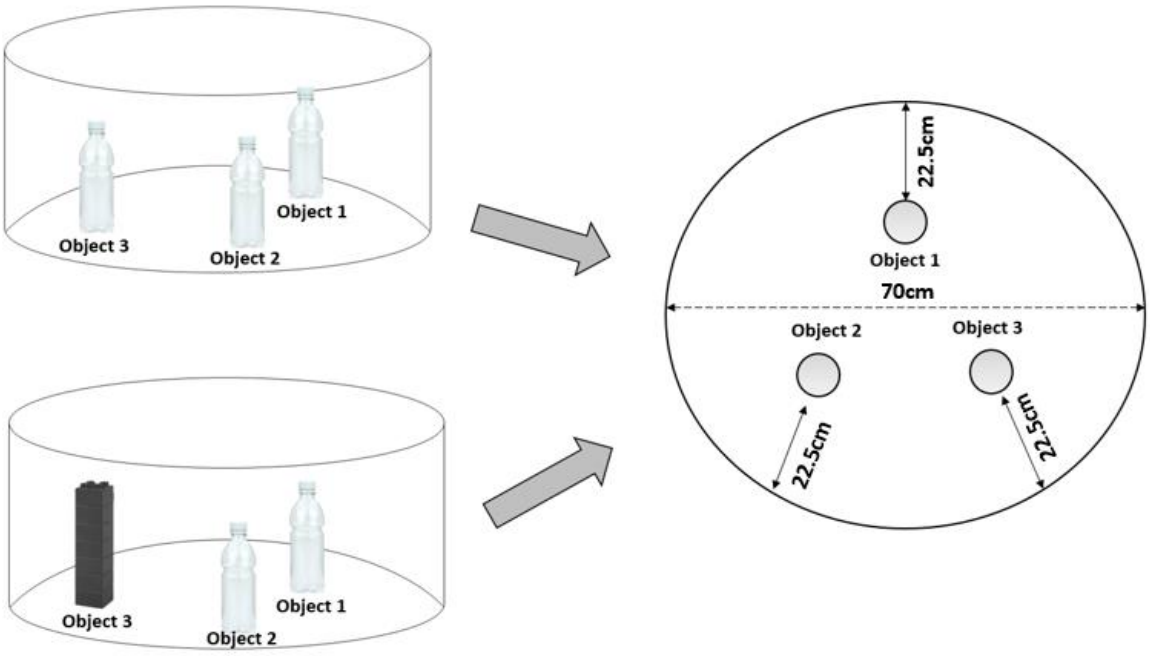


Figure 5.5: Graphical representation of the NOR arena. The left-top image represents the organisation of the object in the sample (familiarization) day. The left-bottom image represents the organisation of the objects in the test day (novel object in the position of object 3). The right image shows the equal distances of the object to the wall of the circular arena.

5.2.5 Brain Extraction

See Chapter 2, section 2.2.5

5.2.6 Statistical Analysis

The SPSS 24.0 statistical package was used to analyse data. Normality was assessed using Shapiro-Wilk test and homogeneity of variance was checked using Levene's test. Behavioural data were analysed using two-factor analysis of variance (Two-way ANOVA), with factors being CFA injection and treatment, or repeated measures ANOVA when appropriate (e.g. when the data were analysed and presented in time bins). *Post hoc* pairwise comparisons were made with Student Newman-Keuls test when appropriate. If data were found to be non-parametric, three transformations were applied, in this order: square root of the data values, log of the data values, and ranking of the data values. Also, it was checked if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular data set being analysed (Thunder et al., 2007). If data were still deemed non-parametric after these transformations and tests, they were analysed using Kruskal Wallis analysis of variance and *post hoc* analysis performed using Dunn's test when

appropriate. When repeated measures were non-parametric distributed, data were analysed using Friedman's and Kruskal Wallis tests followed by Dunn's *post hoc* if applicable. Data were considered significant when $p<0.05$. Data are expressed as group means ± standard error of the mean (SEM) when parametric and as median with interquartile range and min/max values when non-parametric (except for the von Frey data which were non-parametric but presented in timeline graphs as means ± SEM for presentation/readability purposes).

Possible presence of outliers was checked by assessing the distribution of data. In case the data 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.

5.3 Results

5.3.1 CFA induced mechanical allodynia measured by von Frey testing

Friedman's test revealed significant differences among groups both for the contralateral [χ^2 (80) = 41.668, $p<0.001$] and ipsilateral [χ^2 (80) = 55.985, $p<0.001$] hind paw withdrawal thresholds (Figure 5.6). *Post hoc* analysis with Wilcoxon test indicated that the ipsilateral hind paw withdrawal threshold at baseline was significantly higher than on day 1, day 7, day 21, and day 28 ($p<0.001$ for all comparisons). The contralateral hind paw withdrawal threshold at baseline was significantly higher than on day 7, day 21, and day 28. The test also indicated that both ipsilateral and contralateral hind paw withdrawal thresholds were higher on day 1 than day 7 (ipsilateral, Day 7 vs Day 1 $p<0.05$; contralateral, Day 7 vs Day, 1 $p<0.001$) and day 28 (ipsilateral, Day 28 vs Day 1 $p<0.01$; contralateral, Day 28 vs Day 1 $p<0.001$). The contralateral hind paw withdrawal threshold was higher on day 21 compared to day 1 ($p<0.001$). Kruskal Wallis test showed a significant difference among groups on day 1 [χ^2 (9) = 35.069, $p<0.001$], day 7 [χ^2 (9) = 48.980, $p<0.001$], day 21 [χ^2 (9) = 51.601, $p<0.001$], and day 28 [χ^2 (9) = 39.580, $p<0.001$], but not at baseline [χ^2 (9) = 8.236, $p>0.05$] in the withdrawal threshold of the ipsilateral paw. *Post hoc* analysis with Dunn's test indicated significantly lower paw withdrawal thresholds in CFA vehicle-treated animals compared to their No-CFA counterparts on days 1,7,21, and 28 (CFA Vehicle vs No-CFA Vehicle, $^{*}p<0.05$ and $^{**}p<0.01$; Figure 5.5B). The test also indicated lower paw withdrawal thresholds in CFA GW9662 (Figure 5.5H) and PEA (Figure 5.5J)-treated animals compared to their No-CFA counterparts (CFA GW9662 vs No-CFA GW9662, days 7 and 28, $^{\#}p<0.05$; CFA PEA vs No-CFA PEA, days 7 and 21, $^{\#}p<0.05$). Kruskal Wallis did not show any significant difference among groups at baseline [χ^2 (9) = 9.921, $p>0.05$], day 1 [χ^2 (9) = 9.921, $p>0.05$], day 7 [χ^2 (9) = 5.061, $p>0.05$], day 21 [χ^2 (9) = 7.939, $p>0.05$], or day 28 [χ^2 (9) = 6.263, $p>0.05$] in the withdrawal threshold of either the contralateral or ipsilateral paw.

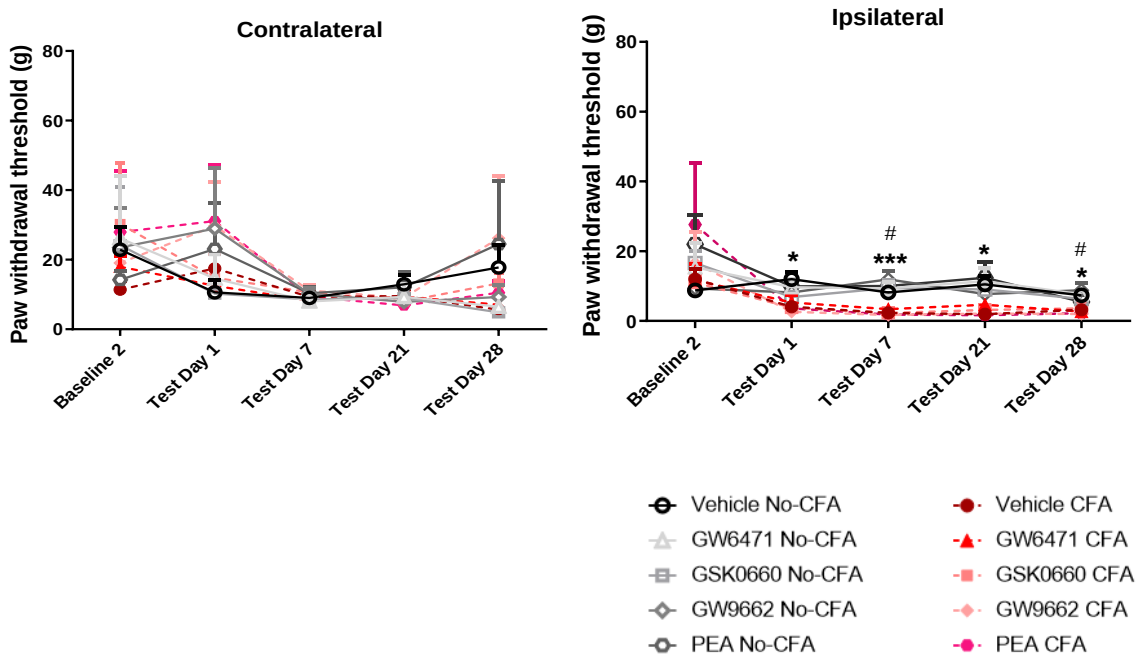


Figure 5.6: Effects of systemic administration of vehicle, selective PPARα (GW6471), PPARβ/δ (GSK0660) and PPARγ (GW9662) antagonists, and PEA on mechanical allodynia in CFA-injected (CFA) rats and control rats that only had needle insertion (No-CFA). Post hoc test indicated significantly lower paw withdrawal thresholds in CFA vehicle-treated animals on days 1,7,21, and 28 (*$p<0.05$ and **$p<0.01$, vs No-CFA Vehicle) in the ipsilateral paw (B). The test also indicated lower paw withdrawal thresholds in CFA GW9662 and PEA-treated animals compared to their No-CFA counterparts ($^{\#}p<0.05$ vs No-CFA GW9662, days 7 and 28; $^{\#}p<0.05$ vs No-CFA PEA, days 7 and 21). The von Frey data which were non-parametric were presented in timeline graphs as means ± S.E.M. for presentation/readability purposes (n=7-8 rats per group).

5.3.2 No effect of PPAR antagonism or PEA on anxiety-related behaviour in the EPM of CFA or non-CFA treated rats

There were no significant effects of treatment [$F(4, 59) = 0.410$, $p>0.05$], CFA [$F(1, 59) = 0.015$, $p>0.05$], or treatment x CFA interaction [$F(4, 59) = 0.835$, $p>0.05$] on the time spent in the open arms of the EPM (Figure 5.7A).

There were no significant effects of treatment [$F(4, 59) = 0.408$, $p>0.05$], CFA [$F(1, 59) = 0.265$, $p>0.05$] or treatment x CFA interaction [$F(4, 59) = 2.047$, $p>0.05$] on the time spent in the close arms of the elevated plus maze (Figure 5.7B).

There were no significant effects of treatment [F (4, 59) = 0.384, p>0.05], CFA [F (1, 59) = 0.010, p>0.05] or treatment x CFA interaction [F (4, 59) = 1.042, p>0.05] on the number of entries into the open arms of the elevated plus maze (Figure 5.7C).

There were no significant effects of treatment [F (4, 59) = 0.940, p>0.05], CFA [F (1, 59) = 0.131, p>0.05] or treatment x CFA interaction [F (4, 59) = 1.203, p>0.05] on the number of entries into the close arms of the elevated plus maze (Figure 5.7D).

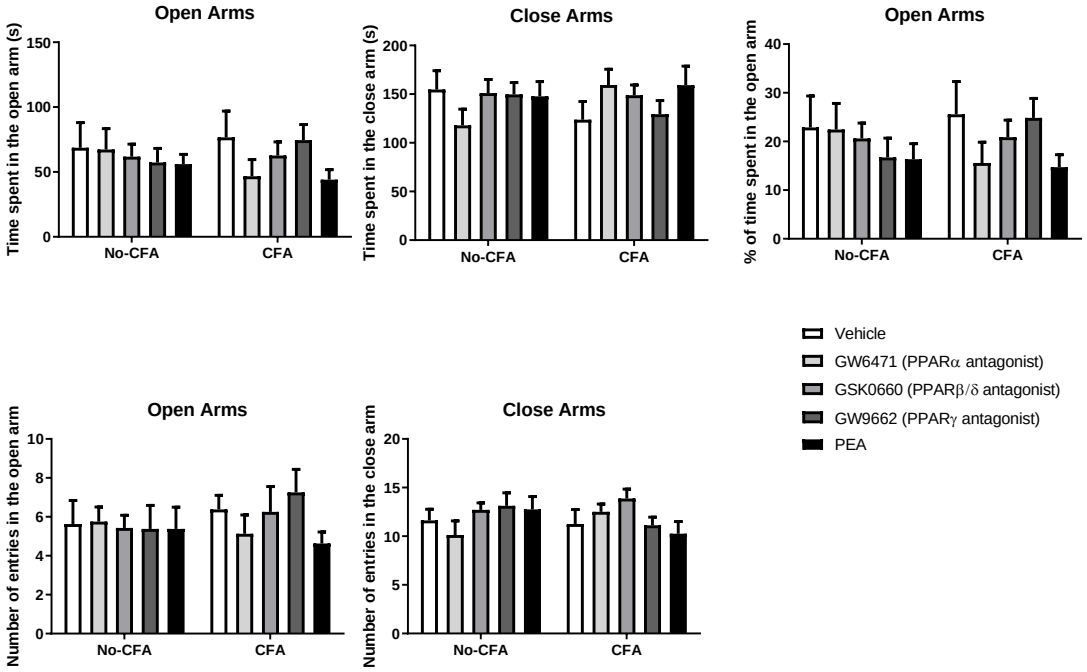


Figure 5.7: Effects of systemic administration of vehicle, selective PPARα (GW6471), PPARβ/δ (GSK0660) and PPARγ (GW9662) antagonists, and PEA on behaviour in the EPM in CFA-injected (CFA) rats and control rats that only had needle insertion (No-CFA). Data are expressed as means ± SEM (n=8 rats per group).

5.3.3 No effect of PPAR antagonism or PEA on anxiety-related behaviour in the OF of CFA or non-CFA treated rats

There were no significant effects of treatment [F (4, 67) = 0.537, p>0.05], CFA [F (1, 67) = 1.364, p>0.05] or treatment x CFA interaction [F (4, 67) = 0.979, p>0.05] on the time spent in the centre zone of the open field arena. (Figure 5.8A)

Kruskal Wallis did not reveal any difference among groups [χ^2 (9) = 9.454, p>0.05] in the time spent in the outer zone of the open field arena (Figure 5.8B)

There were no significant effects of treatment [F (4, 67) = 0.4123, p>0.05], CFA [F (1, 67) = 3.066, p>0.05] or treatment x CFA interaction [F (4, 67) = 0.9724, p=0.071] on the total distance moved by the rats (Figure 5.8C).

There were no significant effects of treatment [F (4, 67) = 1.220, p>0.05], CFA [F (1, 67) = 0.4476, p>0.05] or treatment x CFA interaction [F (4, 67) = 0.8784, p>0.05] on the number of entries into the centre of the open field arena (Figure 5.8D).

There were no significant effects of treatment [F (4, 67) = 1.437, p>0.05], CFA [F (1, 67) = 1.386, p>0.05] or treatment x CFA interaction [F (4, 67) = 0.7996, p>0.05] on the number of entries into the outer zone of the open field arena (Figure 5.8E).

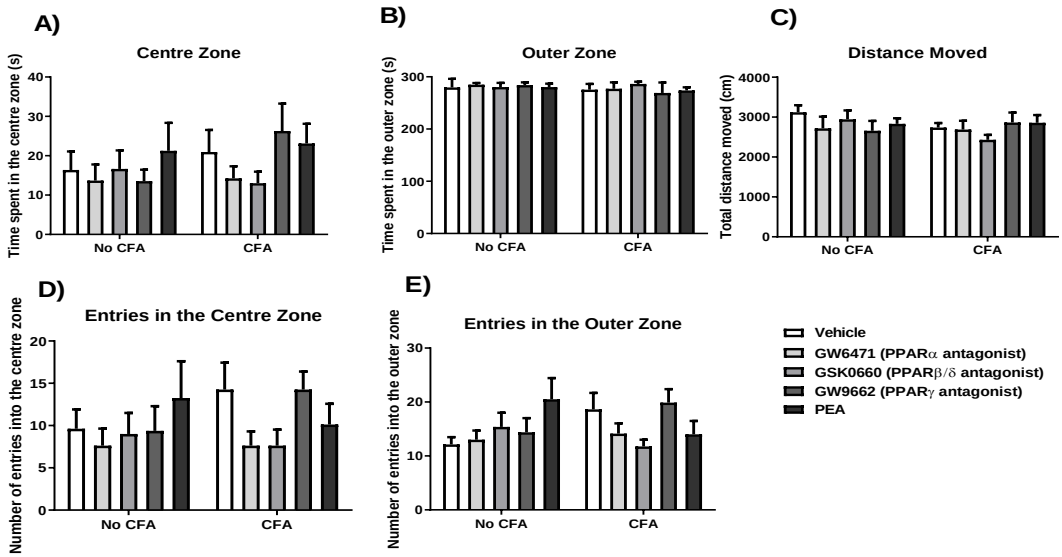


Figure 5.8: Effects of systemic administration of vehicle, selective PPARα (GW6471), PPARβ/δ (GSK0660) and PPARγ (GW9662) antagonists, and PEA on behaviour in the OF test in CFA-injected (CFA) rats and control rats that only had needle insertion (No-CFA). Data are expressed as means ± SEM (A, C, D, and E) or median with interquartile range and min/max (B) (n=8 rats per group).

5.3.4 Trends for an effect of PPAR antagonism or PEA on anxiety-related behaviour in the LDB of CFA treated rats

Kruskal Wallis test did not reveal any difference among groups [χ^2 (9) = 5.060, p>0.05] in the time spent in dark side of the light-dark box (Figure 5.9A).

Kruskal Wallis did not reveal any difference among groups [χ^2 (9) = 6.514, p>0.05] in the time spent in light side of the light-dark box (Figure 5.9B). An analysis of the area under the curve indicated a trend for a decrease in time spent in the light side in CFA-GW6471 treated (p=0.075; Figure 5.9F) and for CFA-GSK0660 treated (p=0.07; Figure 5.9G) rats compared to CFA-Vehicle.

Kruskal Wallis did not reveal any difference among groups [χ^2 (9) = 10.382, p>0.05] in the latency to enter the dark side of the light-dark box (Figure 5.9C).

Kruskal Wallis did not reveal any difference among groups [χ^2 (9) = 11.067, p>0.05] in the number of entries into the light side of the light-dark box (Figure 5.9D).

Kruskal Wallis did not reveal any difference among groups [χ^2 (9) = 12.610, p>0.05] in the number of entries into the dark side of the light-dark box (Figure 5.9E).

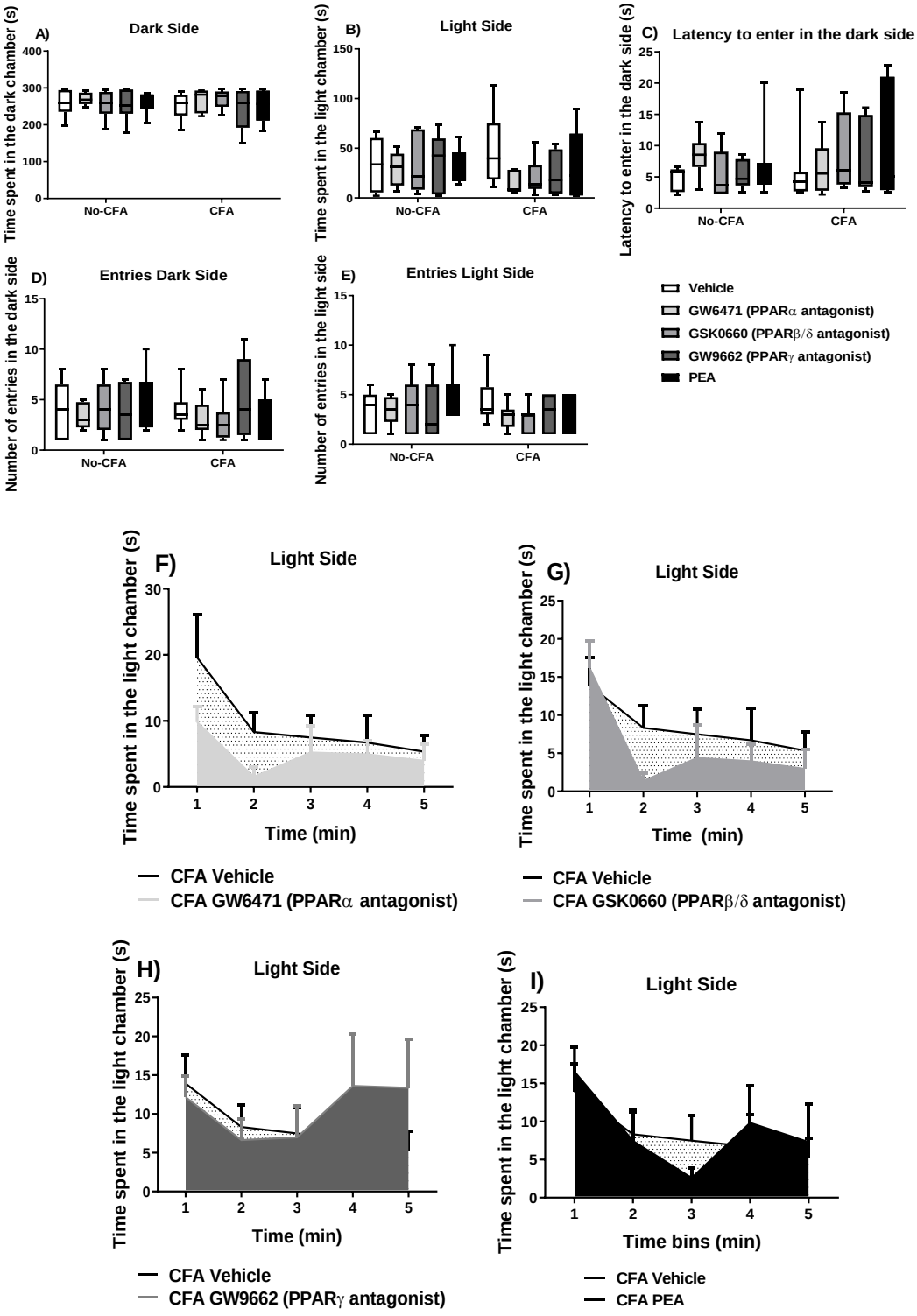


Figure 5.9: Effects of systemic administration of vehicle, selective PPARα (GW6471), PPARβ/δ (GSK0660) and PPARγ (GW9662) antagonists, and PEA on behaviour in the LDB test in CFA-injected or control (No-CFA) rats. The area under the curve indicated a trend for significant decrease of time spent in the light side in CFA-GW6471 treated (p=0.075; F) and CFA-GSK0660 treated (p=0.07; G) rats. Data are expressed as median with interquartile range and min/max (A-E) or means ± SEM (F-I) (n=7-8 rats per group).

5.3.5 Impaired spatial memory by PPARα antagonism in CFA treated rats in the NOR test.

Two-way ANOVA revealed a significant effect of day [F (1, 140) = 50.469, [a]p<0.001] on the percentage of time spent exploring the location of the novel object/object 3 (i.e. preference index; Figure 5.10A). However, *post hoc* analysis with Student Newman-Keuls did not reveal any significant differences between groups. There were no significant effects of treatment [F (4, 140) = 0.772, p>0.05], CFA [F (1, 140) = 2.237, p>0.05], or interactions of treatment x CFA [F (4, 140) = 0.820, p>0.05], treatment x day [F (4, 140) = 0.475, p>0.05], CFA x day [F (1, 140) = 0.553, p>0.05], and treatment x CFA x day [F (4, 140) = 0.414, p>0.05] on the preference index.

Two-way ANOVA revealed a significant effect of CFA [F (1, 62) = 6.006, [£]p<0.05] on the discrimination index, defined as the time exploring the novel object minus the average time exploring the familiar objects (Figure 5.10B). However, *post hoc* analysis with Student Newman-Keuls did not reveal significant differences between groups. There were no effects of treatment [F (4, 62) = 0.535, p>0.05] and the interaction of treatment x CFA [F (4, 65) = 0.924, p>0.05] on the discrimination ratio.

Two-way ANOVA revealed a significant effect of CFA [F (1, 66) = 5.105, [£]p<0.05] on the discrimination index for spatial memory, defined as the difference between the time exploring the novel object (test day) and the time exploring object 3 (sample day) divided by the sum of the time exploring the novel object and the time exploring object 3 (Figure 5.10C). However, *post hoc* analysis with Student Newman-Keuls did not show any further significant differences between groups. There were no significant effects of treatment [F (4, 66) = 1.804, p>0.05] or treatment x CFA [F (4, 66) = 1.233, p>0.05] on the spatial discrimination index. When the data were split by CFA, one-way ANOVA revealed that treatment had a significant effect on the CFA-injected group [F (4, 33) = 3.239, p<0.05]. *Post hoc* analysis with Student Newman-Keuls indicated that GW6471 significantly reduces the discrimination index for spatial memory compared to vehicle-treated rats ([#]p<0.05, vs CFA-Vehicle). There was no significant effect of treatment on the group that did not receive an injection of CFA [F (4, 33) = 0.182, p>0.05].

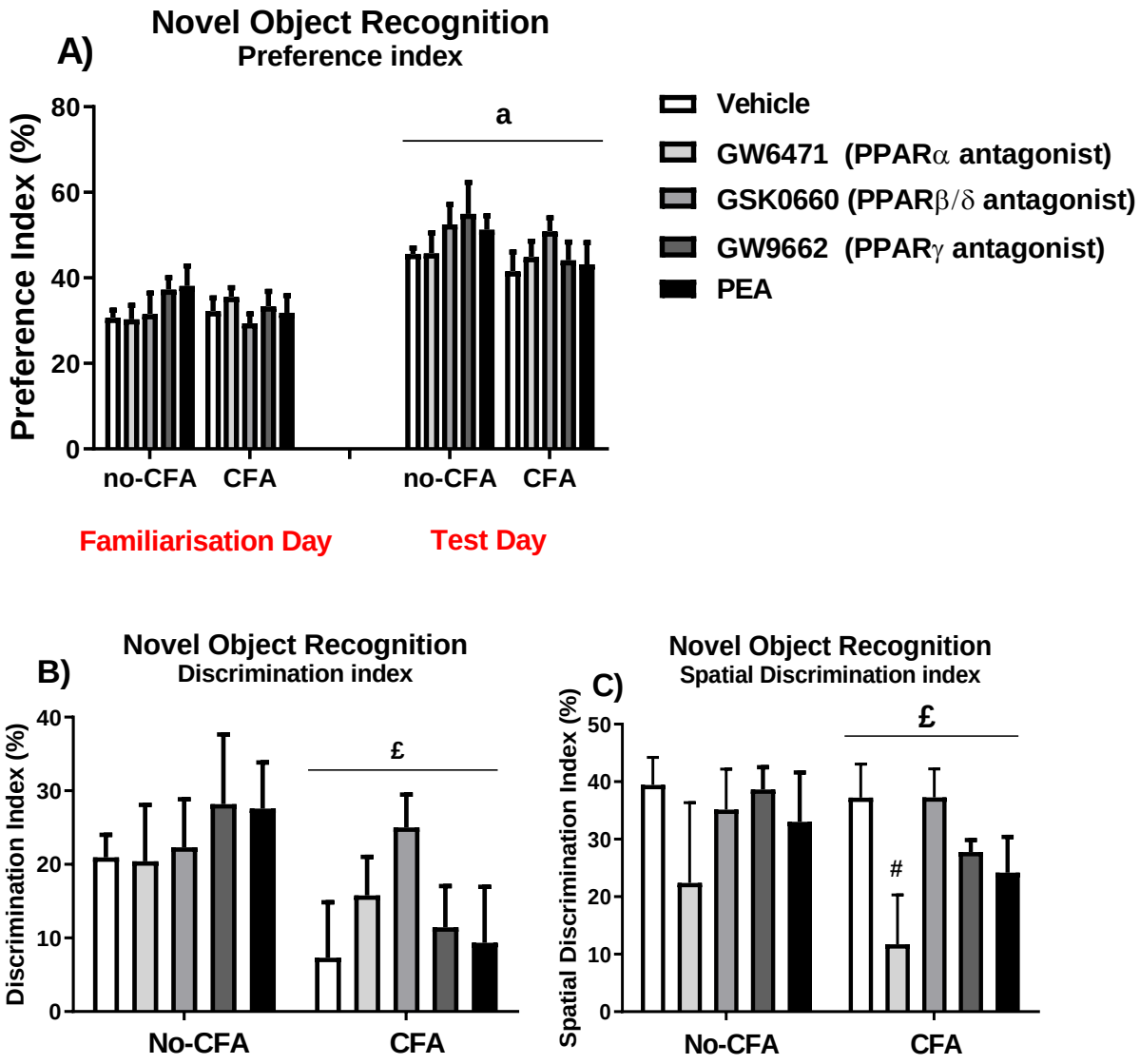


Figure 5.10: Effects of systemic administration of vehicle, selective PPARα (GW6471), PPARβ/δ (GSK0660) and PPARγ (GW9662) antagonists, and PEA on behaviour in the NOR test in CFA-injected (CFA) rats and control rats that only had needle insertion (No-CFA). Two-way ANOVA revealed an effect of day ($^{a}p<0.001$) on the percentage of time spent exploring the novel object compared to object 3 (i.e. preference index = $T_{Object3\ or\ Novel\ Object}$/ ($T_{Object\ 1}$+$T_{Object\ 2}$/2) + $T_{Object3\ or\ Novel\ Object}$; A). CFA ($^{£}p<0.05$; B and C) was also shown to have an effect on the discrimination index (Discrimination index = $T_{Novel\ object}$/ ($T_{Object1}$+$T_{Object2}$/2) + $T_{N)}$ and on the spatial discrimination index (Spatial discrimination index = $T_{Novel\ object}$ - $T_{Object3}$ / $T_{Novel\ object}$ + $T_{Object3)}$. GW6471 significantly reduces the spatial discrimination index compared to vehicle-treated rats ($^{\#}p<0.05$, vs CFA-Vehicle; C). Data are expressed as means ± SEM (n=7-8 rats per group).

5.4 Discussion

The experiment described in this chapter investigated a possible role of PPARs in the modulation of anxiety and cognition in the presence and absence of chronic inflammatory pain. Several studies have indicated that PPAR signalling is involved in regulation of anxiety responses. However, in my experiment, the systemic administration of PPAR antagonists or PEA did not significantly affect the behaviour of rats in the EPM, OF and LDB tests, although a trend for an anxiogenic effect of the blockade of PPARα and PPARβ/δ in the CFA-injected animals was observed in the LDB. The findings described here are in accordance with Panlilio et al. (2009), who reported that the systemic administration of MK886, a PPARα antagonist, did not alter anxiety-like behaviour in the OF test (Panlilio et al., 2009). The lack of significant effect of PPAR antagonism on anxiety behaviour is not in line with what was observed by Domi et al (2016), who demonstrated that the administration of GW9662 had anxiogenic effects in WT mice on the LDB, OF and EPM tests. However, the use of different species may explain the different results. To my knowledge, the present study is the first study to investigate the effects of the blockade of PPARβ/δ in anxiety responses. Both pioglitazone and rosiglitazone, agonists at PPARγ, have previously been shown to elicit anxiolytic-like effects in the LDB, EPM and OF tests (Domi et al., 2016; Guo et al., 2017; Aghaei et al., 2019). To my knowledge only one recent study has investigated the effect of PEA on anxiety-related behaviour and showed that chronic administration of PEA increased sucrose preference and exploration time in the OF test, and these effects were blocked by the PPARα antagonist MK886 (Li et al., 2019). This is not in accordance with what I observed in my experiment, although the authors used a higher concentration of PEA (i.e. 2.5 mg/kg) and only analysed exploratory behaviour and immobility in the OF test.

The effects of PPAR blockade on cognitive tasks is less explored. In the experiment described in this chapter, I have observed that the administration of GW6471 reduced spatial mnemonic performance in the NOR test. Additionally, the administration of PEA did not have any effect on cognitive performance in my experiment. An effect of PPAR activation on learning has previously been described. Mazzola et al (2009) showed that the administration of URB597 (FAAH inhibitor) before the learning trial of a passive avoidance test enhanced the learning of the task, and this enhancement was attenuated by the administration of a PPARα antagonist, MK886. Following this result, they also demonstrated that the administration of a PPARα agonist, WY14643, produced learning

enhancement effects similar to those observed with URB597, and they were also blocked by MK886. Also, a study from Campolongo et al. (2009) indicated that the administration of OEA improved learning of passive avoidance and spatial memory tasks when given immediately post-training and that the actions of OEA were mimicked by the PPARα agonist GW7647 and absent in PPARα null mice. Recently, Ratano et al. (2017) have shown that the cognitive enhancing effects of URB597 are dependent on PPARα, as well as CB_1 and $TRPV_1$ receptors. Pioglitazone administration improved short-term mnemonic performance in mice, an effect most likely mediated through the PPARγ pathway (Masciopinto et al., 2012). However, the data herein did not observe any effect of PPARγ antagonism on NOR. Furthermore, the data here demonstrate a lack of effect of PEA in the NOR task however, the specific effect of PEA on cognition still needs to be explored in greater detail.

The intraplantar administration of CFA resulted in robust mechanical allodynia in the injected paw on days 1, 7, 21 and 28 post-CFA injection. Injection of GW6471, GSK0660, GW9662 or PEA did not alter pain responses at any of these time points. Several studies have demonstrated the involvement of PPAR signalling in the mediation of pain responses in acute and chronic inflammatory models of pain (Okine et al., 2018). Our results do not show any alteration in CFA-induced pain responses following systemic administration of PPAR antagonists, in line with the formalin test data presented in chapter 2. These findings are in accordance with Donvito et al. (2017) and Mansouri et al (2015) who demonstrated that intraperitoneal administration of GW6471 and GW9662 did not affect nociceptive behaviour in the formalin test of tonic, persistent inflammatory pain. Other studies have shown antinociceptive effects of PEA-induced PPAR activation in rodent models of inflammatory (LoVerme et al., 2006; D'Agostino et al., 2009a; Sasso et al., 2012) and neuropathic (LoVerme et al., 2006; Costa et al., 2008; Bettoni et al., 2013; Di Cesare Mannelli et al., 2013, 2015; Guida et al., 2015; Donvito et al., 2016) pain. Therefore, our results for PEA diverge to some extent from those reported in the literature, although important to note that, to date, no studies have investigated the effects of PEA in the CFA model. Moreover, due to the organisation of the experimental design, the rats went through anxiety or cognitive tests before pain assessment, which may have affected pain responsivity to PPAR modulation.

In the present study, CFA injections did not affect anxiety responses in the EPM, OF or LDB tests. This result is at odds with other studies showing CFA-induced anxiety. For instance, Parent et al. (2012) have shown that intraplantar injection of CFA had anxiogenic effects in the EPM and OF, but not LDB, tests in rats. Although the authors used the same tests and the same rat strain used herein, they chose different time points post-CFA to run their tests (i.e. day 28-30 versus day 21 in the present study). Hofmann et al. (2017) have seen CFA-induced anxiety behaviour in the LDB and EPM tests with mice. Other studies have also shown increased anxiety-like behaviours in mice following intraplantar CFA injection in EPM and OF tests (Shi et al., 2010; Wang et al., 2015; Guo et al., 2016; Yue et al., 2018). Similarly, intraplantar injections of CFA also resulted in increased anxiety in mice in the place escape/avoidance paradigm (PEAP) and elevated zero maze tests (Refsgaard et al., 2016). The injection of CFA into the temporomandibular joint also produced anxiety-like behaviours in the EPM and LDB tests (do Nascimento and Leite-Panissi, 2014) in Wistar rats. In summary, these studies indicate an anxiogenic effect of CFA injections. However, differences in the animal model (i.e. rats vs mice, SD vs Wistar) and the time points at which the tests were performed could explain the discrepant results seen in my experiment.

Furthermore, in the present experiment, CFA-injected rats exhibited impaired recognition and spatial mnemonic performance in the NOR test. Interestingly, administration of the PPARα antagonist GW6471 further impaired spatial memory in CFA-treated rats, but not in non-CFA injected controls. These results are in accordance with previous studies that have indicated CFA-induced cognitive deficits. Yang et al (2014) have demonstrated that CFA injection impairs the learning of tone-footshock, but not context-footshock, association in mice. Moreover, intraplantar CFA injections in day 1 and day 8 postnatal rats impaired cognitive performance in the NOR and spatial test by Morris water maze (Amaral et al., 2015). Similarly, injections of CFA in day 2 postnatal also impaired spatial memory in the same test (Li et al., 2005). Importantly, similar to what I have found in my experiment, another study shows that morphine has differential effects on memory performance of rats depending on the pain status; specifically, that while CFA itself had little effect on spatial memory, morphine injections in pain-free animals impaired spatial memory and morphine administration in CFA-treated rats had no effects on mnemonic performance (Baiamonte et al., 2013). Intraplantar injections of CFA were shown to increase PPARα expression in the spinal cord of rats (Benani et al., 2004). Further studies would be

required to elucidate the mechanisms underpinning the differential effect PPARα blockade in CFA-versus non CFA-injected rats, in particular in key regions involved in cognitive responses.

In conclusion, these results indicate a modulatory effect of chronic inflammatory pain on cognitive processing, but not on innate anxiety-related responses. Moreover, in the presence of chronic inflammatory pain, blockade of PPARα impaired spatial memory and tended to increase anxiety-related responses in the LDB test. PPARβ/δ and PPARγ blockade nor PEA did not modulate cognition or anxiety, either in presence or in the absence of pain. The blockade or activation of these receptors does not appear to modulate mechanical allodynia evoked by CFA injection.

6. General Discussion

Anxiety disorders are among the most prevalent mental diseases worldwide. According to the World Health Organisation in the document entitled *"Depression and other common mental disorders"* from 2017, 3.6% (264 million people) of the global population is affected by anxiety disorders. Chronic pain is also an unmet clinical problem which afflicts one in five Europeans and is frequently associated with mood disorders. A substantial number of studies show that patients suffering with chronic pain have higher prevalence of co-morbidity with anxiety disorders and depression (Demyttenaere et al., 2007; Asmundson and Katz, 2009; Velly and Mohit, 2018). Pain and fear/anxiety share a reciprocal relationship whereby they can modulate one another. FCA is an example of how fear can modulate pain responses. In this phenomenon, pain is suppressed upon presentation of a stressful or fearful stimulus. Likewise, anxiety can exacerbate painful experiences. People suffering with anxiety disorders report higher pain scores than healthy controls (Pompili et al., 2012). Pain also affects anxiety and fear responses. Patients with chronic pain are twice as likely to develop phobias (Pereira et al., 2017) and PTSD symptoms tend to be more pronounced in patients with chronic pain (Asmundson et al., 2002). The neurobiological mechanisms that underlie the interaction of pain and anxiety/fear are not completely understood. A better understanding of the neurobiology of this interaction could inform the development of new pharmacotherapies for the management of pain and its comorbidity with affective disorders. In this regard, it is important to investigate the participation of potential endogenous modulators, such as PPARs in pain, fear/anxiety responses, and pain and fear interactions.

The body of work described in this thesis aimed to add to the current knowledge on inflammatory pain, FCA and the influence of inflammatory pain on PPAR-mediated modulation of conditioned fear, innate anxiety and cognition. Here, I will summarise the most significant behavioural and neurochemical findings and discuss how these results contribute to improve our understanding on the neurobiology of pain, fear and anxiety, FCA, and cognition. Finally, I will point out some limitations of the experiments and highlight some areas that in my opinion deserve further investigation in the future.

The main findings of the body of work reported and discussed in this thesis are:

(1) The systemic administration of PPARα and PPARβ/δ antagonists prolonged, while the systemic administration of a PPARγ antagonist enhanced, the expression of freezing behaviour in FC rats in the presence of formalin-evoked nociceptive tone.
(2) The systemic administration of PPARα, PPARβ/δ or PPARγ antagonists had no effect on either formalin-evoked nociceptive behaviour or FCA.
(3) Intra-BLA administration of a PPARα antagonist prolonged, and of a PPARγ antagonist increased, expression of freezing behaviour in FC rats in the presence of formalin-evoked nociceptive tone. These results were associated with increased tissue levels of dopamine in the right BLA.
(4) Intra-BLA administration of PPARα, PPARβ/δ or PPARγ antagonists had no effects on either formalin-evoked nociceptive behaviour or FCA.
(5) Intra-BLA administration of PPARα, PPARβ/δ or PPARγ antagonists increased freezing expression in NFC rats in the absence of formalin-evoked nociceptive tone. This result was associated with increased tissue levels of dopamine in the right BLA.
(6) Intra-CeA administration of PPARα, PPARβ/δ or PPARγ antagonists had no effects on formalin-evoked nociceptive behaviour, FCA or conditioned fear in the presence or absence of nociceptive tone.
(7) The systemic administration of a PPARα antagonist impaired spatial recognition memory in the presence, but not the absence, of chronic inflammatory pain, and tended to have an anxiogenic effect. The systemic administration of PPARβ/δ antagonists also resulted in trends for an anxiogenic effect.
(8) Spatial recognition memory was impaired in the rat CFA model of chronic inflammatory pain.

In chapter 2, I aimed to investigate the effects of systemic administration of PPARα, PPARβ/δ and PPARγ antagonists on acute inflammatory pain responses, FCA and conditioned fear in the presence of nociceptive tone. The results demonstrate that pharmacological blockade of PPARα or PPARβ/δ prolonged freezing duration in rats that had also received intra-plantar injection of formalin. These findings were associated with increased levels of dopamine in the right CeA of FC animals only. In addition, the administration of a PPARγ antagonist increased the expression of freezing. The antagonists

did not have any effect on formalin-evoked nociceptive behaviour or FCA. Although no other studies have specifically examined the participation of PPARs in fear responses and, consequently, in the extinction process, a few studies explored the role of PPARs in memory formation. Mazzola et al. (2009) have shown that intraperitoneal administration of WY14643, a PPARα synthetic agonist, enhanced memory acquisition and Campolongo et al. (2009) have shown that post-training administration of the endogenous PPAR ligand OEA enhanced memory consolidation in both spatial and passive-avoidance learning tests, effects that were abolished in mutant mice lacking PPARα. Importantly, a recent study have shown that PPARα knockout (PPARα KO) mice had enhanced fear learning compared to WT counterparts (Chikahisa et al., 2019). In my experiment, the prolongation of freezing duration by GW6471 was associated with increased levels of GABA and glutamate in the ventral hippocampus and in the BLA, and increased levels of dopamine in the CeA. We suggested that the alteration in extinction observed upon the administration of PPAR antagonists is mediated by changes in the levels of these neurotransmitters. Thus, the results reported in chapter 2 indicate a possible modulatory role of PPARs in fear responses, more specifically in the short-term extinction of fear memories. However, it is important to stress that these effects were observed in the presence of nociceptive tone. A limitation of the work in this chapter is the lack of a control group that does not have any nociceptive stimulus. As seen in chapters 3 and 5, the presence of pain may impact the outcome of PPAR blockade on fear or anxiety-related responses. Future research should explore the effects of systemic administration of antagonists at PPARs in conditioned fear in the absence of nociceptive tone. Another limitation of the protocol used to examine effects of PPARs on pain and FCA is the duration of the trial (i.e. 15 minutes). The short exposure, although chosen based on previous studies showing that this is the peak time for expression of FCA and appropriate to observe possible attenuations in FCA, limits the ability to detect a possible enhancement or prolongation of FCA. Therefore, future investigations should consider increasing the exposure time in order to allow the observation of augmentations in FCA (as was done in subsequent chapters). In my experiments, prolonged freezing expression was associated with increased levels of dopamine in the right CeA. A recent study revealed that dopamine and its metabolites were increased in the amygdala of PPARα KO mice and the systemic administration of a dopamine antagonist attenuated the enhanced fear learning observed in PPARα KO animals (Chikahisa et al., 2019). Hence, a possible modulatory role of PPARs

on dopamine release in the amygdala, particularly in the CeA, may prove to be an important target for future research.

Because the amygdaloid complex is a key region for fear and anxiety-related behaviours and is also important for pain processing, I followed the findings of chapter 2 by investigating the effects of the blockade of PPARs expressed in two of its subnuclei – the BLA and the CeA – on acute inflammatory pain, FCA and conditioned fear in the presence or in the absence of nociceptive tone (Figure 6.1). Both the BLA and the CeA were shown in previous studies to be important in fear expression and extinction (see section 1.3.1.1) and pain processing (section 1.2.2). The results of chapter 3 were in accordance to what was seen in chapter 2: in the presence of nociceptive tone, the blockade of PPARα in the BLA prolonged freezing duration while the blockade of PPARγ expressed in the BLA enhanced expression of freezing. Interestingly, these results were also associated with increased tissue levels of dopamine in the right BLA. Importantly, these findings seem to be exclusive to the BLA, because in chapter 4 we demonstrated that the intra-CeA administration of PPARs antagonists did not affect fear responses. As stated above, while recent studies have demonstrated a role for PPARα in the enhancement of fear learning (Mazzola et al., 2009b; Chikahisa et al., 2019), this was the first experiment, to my knowledge, to examine the participation of PPAR signalling in the BLA in conditioned fear responses. The findings herein indicate a possible role of PPARα and PPARγ expressed in the BLA in fear responses and/or associative extinction learning in the presence of nociceptive tone. In chapters 2 and 3 that the prolongation in freezing duration observed upon blockade of PPARs in FC rats and in the presence of a nociceptive tone was likely to be associated with impaired short-term within trial extinction of memories as a consequence of a deficiency in the formation of new memories. Hence, the blockade of PPARs could be associated with an impairment in memory acquisition. Supporting this hypothesis, other studies have demonstrated a modulatory role of PPARs in mnemonic performance. Administration or manipulation of the levels of endogenous ligands at PPARs has been shown to enhance cognitive performance (Campolongo et al., 2009a; Goonawardena et al., 2011; Morena et al., 2014; Kramar et al., 2017; Rueda-Orozco et al., 2017; Scuderi et al., 2018; Segev et al., 2018; Zimmermann et al., 2018; Boccella et al., 2019), and a few studies indicate a possible modulatory effect of PPARs in fear learning processes. For instance, Mazzola et al (2009) have shown that the administration of URB597 (FAAH inhibitor) before the learning trial of a passive avoidance test enhances the learning of the task and this enhancement was

mimicked by the administration of a PPARα agonist, WY14643. Additionally, a study from Campolongo et al. (2009) indicated that the administration of OEA improved learning of passive avoidance and spatial memory tasks and that the actions of OEA were mimicked by the PPARα agonist GW7647 and absent in PPARα null mice. Recently, Ratano et al. (2017) have shown that the cognitive enhancing effects of URB597 were dependent on PPARα, and PPARα knockout mice showed enhanced fear learning compared to their WT counterparts (Chikahisa et al., 2019).

In the absence of nociceptive stimulus, the intra-BLA administration of PPARα, PPARβ/δ and PPARγ increased freezing expression in NFC rats. Once more, these results were associated with increased levels of dopamine in the right BLA of rats. These findings are also exclusive to the BLA, since in chapter 4 we have shown that the intra-CeA administration of PPAR antagonists did not elicit any alteration in behaviour in the absence of a nociceptive tone. Recent studies have indicated a possible role of PPARs in anxiety. Youssef et al. (2019) have shown that the administration of a PPARγ antagonist blocked the anxiolytic effect of beta-caryophyllene and repeated stress decreased PPARγ expression in the amygdala (Liu et al., 2018). PPARγ blockade or knockout was shown to have anxiogenic effects in mice and intra-amygdala injections of pioglitazone (PPARγ agonist) were shown to reduce stress-induced anxiety behaviour in rats (Domi et al., 2016). In my experiment, NFC rats that received intra-BLA injections of PPAR antagonists in the absence of nociceptive tone had increased levels of freezing, comparable to their FC counterparts. Thus, the blockade of these receptors in the BLA increased innate anxious state. Supporting this hypothesis, other studies have demonstrated a modulatory role of PPARs in anxiety. Brain levels of endogenous ligands at PPARs have been shown to be increased in response to stress or anxiety (Bluett et al., 2014; Hillard, 2018) and the plasmatic level of OEA is significantly lower in PTSD patients compared to controls (Wilker et al., 2016). Genetic deletion of neuronal PPARγ has anxiogenic effects in mice (Domi et al., 2016). In this same investigation, the authors showed that systemic and intra-amygdalar injections of pioglitazone (PPARγ agonist) reduced stress-induced anxiety behaviour in rats, and that these effects were blocked by the administration of the PPARγ antagonist GW9662, although GW9662 alone did not alter anxiety-related behaviour. In addition, both rosiglitazone (Guo et al., 2017) and pioglitazone (Aghaei et al., 2019) were shown to have anxiolytic effects on the open field test in rats. Our results support the findings reported

above, demonstrating that PPAR signalling in the BLA may modulate anxiety-related behaviour.

In the intra-CeA experiments (Chapter 4), in which the administration of PPAR antagonists had no effects on the measured parameters, the possibility that the dose administered may not have been adequate to sufficiently block the receptors must be considered. However, these same doses were shown to have effects in the intra-BLA studies described in chapter 3. Additionally, other studies have shown that these doses of the PPARα and PPARγ antagonists elicit behavioural changes (Okine et al., 2014; Domi et al., 2016). Therefore, I believe that it is unlikely that the drug doses chosen were not sufficient. However, more studies exploring the chosen dose for PPARβ/δ would be desirable.

Importantly, a main observation of these experiments is the differential effect of the blockade of PPAR signalling on fear responding, dependent on the presence or absence of pain. In the presence of formalin-evoked nociceptive tone, the neurochemical alterations associated with PPAR blockade appear to be enhanced. I propose that PPARs act to increase dopaminergic release within the BLA, and this effect is augmented in the presence of pain. Accordingly previous studies have shown that PPARs (Chikahisa et al., 2019) and pain (Wood, 2006; Kato et al., 2016) increase dopamine levels in the brain. Alternatively, the blockade of PPARs may alter the endocannabinoid-mediated modulation of the dopaminergic system. The endocannabinoid system has been shown to modulate dopamine release in the nucleus accumbens (French et al., 1997; Tanda et al., 1997; Oleson et al., 2012). Therefore, the blockade of PPARs may shunt binding of their endogenous ligands (i.e. mainly AEA, PEA and OEA) to other receptors (i.e. CB_1, TRPV1, GPR55) that may in turn modulate the dopamine-mediated facilitation of fear responses, an effect which may be enhanced in the presence of pain. The activation of GPR55 by PEA in the VH, for instance, was shown to increase dopaminergic neuronal activity in the VTA (Kramar et al., 2017). Future studies should explore the effects of pain on dopaminergic release within the BLA, and a possible modulatory effect of the presence of a nociceptive tone on PPAR-mediated enhancement in fear-responses through the dopaminergic system. Moreover, alternative binding sites for the endogenous ligands of PPARs could be targeted in future investigations on this modulatory role of pain in fear expression. Regarding the limitations of the work presented in chapter 3, more sessions of extinction could reveal whether PPARs in the BLA act not only in the within-session, but in the complete extinction process of fear memories. It would be interesting if future studies examined the effects of PPAR blockade on long-

term fear extinction by adding extinction trials to the protocol used in my work. Another issue for future investigation is the participation of PPAR signalling in the BLA in the formation, rather than in the extinction, of fear memories by altering the time-point of the microinjections. Moreover, in both experiments, the effects were associated with increased levels of dopamine in the right BLA; this is an interesting finding and a good target for future investigations. Importantly, the results here reported are only seen in the BLA; the blockade of PPARs expressed in the CeA did not affect conditioned fear responses either in the presence or in the absence of a nociceptive tone. Therefore, both the presence or absence of pain and the region of the amygdala targeted were important variables when investigating the participation of PPARs in fear responses.

It must be noted that the blockade of PPARs expressed in the BLA or in the CeA did not affect formalin-evoked nociceptive behaviour or FCA. These findings are also in accordance with what we observed in chapter 2, where systemic administration of PPARs did not alter pain responses or FCA. To my knowledge, no other studies have investigated the role of the PPAR signalling within the amygdala in inflammatory pain responses or FCA. As mentioned in chapters 3 and 4, other groups have revealed that systemic administration of PPAR agonists has antinociceptive effects, and systemic administration of antagonists at PPARs do not seem to affect pain responses (Donvito et al., 2017; Mansouri et al., 2017). FCA has been previously shown to be associated with increased levels of AEA, an endocannabinoid which also binds to PPARs, in the BLA (Rea et al., 2013) and a strong trend for increased tissue levels of PEA and OEA, endogenous ligands of PPARs, in the BLA. However, the blockade of PPARs in the BLA and CeA do not seem to alter the expression of FCA.

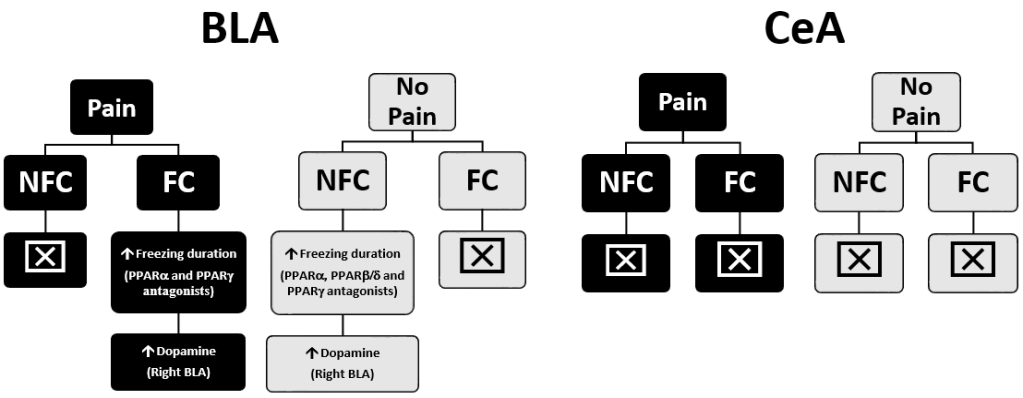


Figure 6.1: Schematic summary of the key results seen in chapters 3 and 4.

The results seen in chapter 3, in which the microinjections of PPARα, PPARβ/δ or PPARγ antagonists into the BLA increased freezing expression in NFC rats, indicated a possible enhancement of innate anxiety and, therefore, a participation of PPAR signalling in anxiety responses. Additionally, we discussed in chapters 2 and 3 that the prolongation of freezing observed upon blockade of PPARα in FC rats and in the presence of nociceptive tone (chapter 2 and 3) and PPARβ/δ (chapter 2) was likely to be associated with impaired short-term within trial extinction of memories as a consequence of a deficiency in the formation of new memories. Hence, the blockade of PPARs could be associated with an impairment in memory acquisition. Thus, in order to assess if the effects on conditioned fear seen in chapters 2 and 3 and the anxiety-like results observed in chapter 3 could be explained by a modulatory effect of PPARs on anxiety and cognition, I conducted one final experiment in which I examined the effects of the systemic administration of PPARα, PPARβ/δ, or PPARγ antagonists and PEA, a PPAR agonist, on anxiety and cognition. Importantly, because of the differential effect of PPAR blockade in the presence or absence of a pain stimulus seen in chapter 3, we evaluated the participation of PPARs in anxiety and cognitive performance in the presence and absence of CFA-induced chronic inflammatory pain.

The results reveal that the systemic administration of PPAR antagonists did not significantly affect behaviour of rats in the EPM, OF and LDB tests, although a trend for an anxiogenic effect of PPARα and PPARβ/δ blockade in the CFA-injected animals was seen. This finding is in line with what was observed by Domi et al (2016), who demonstrated that the administration of GW9662 alone did not alter anxiety-related responses. Similarly, systemic

administration of MK886, a PPARα antagonist, did not alter anxiety-like behaviour in the open field test (Panlilio et al., 2009). In my experiment, I have seen that the administration of the PPARα antagonist GW6471 reduced spatial mnemonic performance in the NOR test. These results are in line with other studies showing that PPARα agonists improved learning in different mnemonic tasks (Campolongo et al., 2009; Mazzola et al., 2009). Future investigations should aim to further explore the effects of PPAR blockade at other critical time points of the NOR test (i.e. formation of the memory in the sample phase) and other mnemonic tasks (i.e. associative instrumental response, Morris water maze, T-maze test, *etc*), and investigate the neurochemical mechanisms behind these effects. Equally important was the finding that intraplantar CFA injections reduced spatial recognition memory performance in rats. This finding is in accordance with a vast body of work showing that the presence of pain affects cognitive abilities (Moriarty et al., 2011, 2016; Moriarty and Finn, 2014). Future studies could explore this issue further by analysing neurophysiological changes associated with this impairment in cognition and use pharmacological tools to better explore the neurochemistry/neurobiological mechanisms involved. Moreover, techniques such as optogenetics could also help to unveil circuitries involved and add to the body of knowledge on the subject.

The exact neurochemical mechanisms that underlie the modulation of fear, anxiety and cognitive responses by PPARs still remains to be elucidated. In my experiments, the dopaminergic system in the amygdala was repeatedly identified as a potential candidate. One study have shown that dopamine and its metabolites were increased in the amygdala of PPARα knockout (PPARα KO) mice, and intraperitoneal injections of a D1 antagonist attenuated the enhanced fear learning observed in PPARα KO mice. Thus, the modulation of the dopaminergic system by PPARs and how this modulation affects fear extinction, anxiety responses and cognitive performance provide a good starting point for future research. Other neurotransmitters were also shown to be altered by PPAR blockade, although not as consistently as dopamine. For instance, tissue levels of GABA and glutamate were shown to be increased in the BLA after systemic administration of PPARα and PPARβ/δ antagonists. Therefore, future investigations on the interaction between PPARs and the GABAergic and glutamatergic system would add to the current body of work on the PPAR signalling system and inform the identification of novel therapeutic targets for pain, anxiety, cognitive impairment and their comorbidity.

In conclusion, the findings of the studies described in this thesis have indicated that the blockade of PPAR signalling, particularly PPARα and PPARγ expressed in the BLA, prolong or increase freezing in the presence of nociceptive tone; moreover, these results are associated with increased tissue levels of dopamine in the right BLA. In the absence of a pain stimulus, the blockade of PPARs in the BLA increases freezing expression in NFC rats, indicating a possible modulatory role of PPARs in innate anxiety which was also associated with increased levels of dopamine in the BLA. These findings point to a differential effect of the blockade of PPARs expressed in the BLA on fear and anxiety responses in the presence or absence of a nociceptive stimulus. Finally, the systemic administration of a PPARα antagonist impaired spatial memory of rats in the presence of a chronic inflammatory nociceptive stimulus (CFA). The systemic, intra-BLA or intra-CeA administration of PPAR antagonists did not alter pain behaviour in the models tested herein and did not have any effect on FCA. The systemic administration of PPAR antagonists and agonist did not affect anxiety responses either in the presence or in the absence of a nociceptive tone.

The work presented in this thesis has added to the current knowledge on the involvement of the PPAR signalling system in nociceptive responses. Additionally, it has, for the first time, revealed an important modulatory role of PPARs in the fear response, and further contributed to research on the role of PPARs in anxiety. It has also added to the body of knowledge on the involvement of the PPAR signalling system in cognition, mainly in recognition mnemonic performance. Furthermore, the results of the experiments in this thesis have also contributed to research on the modulatory effect of pain on cognitive and fear/anxiety responses.

Appendices

Appendix A

Table A.1: Summary of pharmacological studies investigating the role of PPARs in animal models of pain.

Type of Pain	Model	Drug	Dose	Route of Administration	Animal	Outcome	Reference
Inflammatory	Carrageenean	PEA and GW7647 (PPARα agonist)	2 μl/mouse (i.c.v)0 3 μl (spinal)	i.c.v and spinal	Swiss Mice	Reduced hyperalgesia in mice via inhibition of pro-inflammatory signalling in the carrageenan model of inflammatory pain. The results were mimicked by the PPARα agonist (GW7647)	D'agostino et al, 2009
Other	-	PEA	3 and 6 nmol	Intra-vlPAG	Wistar Rats	Reduced thermo-nociceptive threshold , as well as on/off cell activity in the rostro-ventromedial medulla (RVM)	De Novellis et al, 2012
Inflammatory	Subcutaneos carrageenean	PEA	200,400 and 800 μg/ml	subcutaneos	Wistar Rats	The treatment with PEA has reduced allodynia evaluated by Von Frey	De Filippis et al, 2011
Inflammatory	Carrageenean	PFOA (PPARα agonist) and rosiglitazone (PPARγ agonist)	100 mg/kg	Systemic	Sprague-Dawley Rats	Pretreatment with either drugs inhibited carrageenan-induced edema in a dose-dependent manner, and also reduced carrageenan-induced hyperalgesia	Taylor et al, 2002
Inflammatory	Carrageenean	Rosiglitazone and 15d-PGJ2 (PPARγ agonist)	0-5 - 50 mg (Rosiglitazone) and 50 - 200 mg (15d-PGJ2)	i.c.v	Sprague-Dawley Rats	ICV administration of the drugs dose-dependently reduced behavioural withdrawal responses to noxious heat. The administration of antagonists (BADGE and GW9662) reversed the anti-hyperalgesic effects.	Morgenweck et al, 2010

Inflammatory	Carrageenean and Collagen-induced arthritis	Adelmidrol (PEA analogue)	10 mg/kg	intraperitoneal	Sprague-Dawley Rats	The administration of Adelmidrol produced a signficant inhibition in the development of carrageenean-induced and collagen-induced thermal and mechanical allodynia. This anti-allodynic effect was reversed by GW9662 (PPARg antagonist)	Impellizzeri et al, 2016
Inflammatory	Carrageenean	URB597 (FAAH inhibitor)	25 and 100 μg	intraplantar	Sprague-Dawley Rats	The administration of URB597 attenuated the hyperalgesia induced by carrageenean. GW6471 (PPARa antagonist) reversed this effect.	Jhaveri et al, 2008
Inflammatory	Carrageenean	GW0742 and ATRA(PPARβ/δ agonist)	0.1 mg/kg (GW0742) and 5 mg/kg (ATRA)	intraperitoneal during 4 days	Wistar Rats	The administration of both drugs has reduced mechanical and thermal hyperalgesia induced by carrageenean. The co-treatment with a PPARb/d antagonist (GSK0660) has blocked the effects of the drugs.	Gill et al, 2013
Neuropathic	Chronic constriction injury of sciatic nerve (CCI)	PEA	10 mg/kg	intraperitoneal	Murine	The administration of PEA has reduced thermal hyperalgesia and mechanical allodynia; these effects were mediated by PPARg	Costa et al, 2008
Neuropathic	Chronic constriction injury of sciatic nerve (CCI)	PEA	10 mg/kg	intraperitoneal for seven days	C57BL/6J Mice	The administration of PEA has reduced thermal hyperalgesia and mechanical allodynia	Bettoni et al, 2013
Neuropathic	Spared Nerve Injury	15d-PGJ2 and rosiglitazone (PPARγ agonists)	25 μg, 50 μg, 100 μg and 200 μg (15d-PGJ2) amd 25 μg, 50 μg and 100 μg (rosiglitazone)	intrathecal	Sprague-Dawley Rats	The treatments with the drugs (dose of 100 μg) has decreased mechanical and cold hypersensitivity. The concomitant treatment with PPARg antagonist (BADGE) has reversed these effects	Churi et al, 2008

Neuropathic	Spared Nerve Injury	Pioglitazone (PPARγ agonist)	1,3 and 10 mg/kg per day during 7 days (ip) 0, 0.3, 3.0, 30.0 mg/kg daily during 7 days (included in the diet)	intraperitoneal and oral	Sprague-Dawley Rats	The treatment with pioglitazone had anti-allodynic and anti-hyperalgesic effects and they were reversed by the PPARg antagonist (GW9662)	Morgenweck et al, 2013
Neuropathic	Spared Nerve Injury	Pioglitazone (PPARγ agonist)	2 and 10 mg/kg (i.p.) and 0-300 μg (i.t.)	intraperitoneal and intrathecal	Sprague-Dawley Rats	Treatment has rapidly reduced hyperalgesia induced by SNI; the administration of GW9662 (PPARg antagonist) has reversed the effects)	Griggs et al, 2015
Neuropathic	Diabetic-induced hyperalgesia	Pioglitazone (PPARγ agonist)	30 mg/kg/day	oral (diet)	ZL and ZDF rats	Treatment has recuded mechanical and thermal(hot and cold) hyperalgesia in diabetic rats	Griggs et al, 2016
Neuropathic	Silk suture thread of the sciatic nerve	Pioglitazone (PPARγ agonist)	1-25 mg/kg	oral (diet)	ICR Mice	The treatment has attenuated tactile allodynia	Maeda et al, 2008
Neuropathic	Partial sciatic nerve ligation	Rosiglitazone (PPARγ agonist)	3 and 10 mg/kg	intraperitoneal and local	C57BL6 Mice	Systemic rosiglitazone treatment early in the course of progressive inflammation ameliorated tactile allodynia	Takahashi et al, 2011
Neuropathic	Diabetic-induced hyperalgesia	PEA	30 mg/kg	intraperitoneal	Mice	Treatment with PEA relieves mechanical allodynia	Donvito et al, 2015
Neuropathic	Paclitaxel-induced allodynia	PEA and GW6471 (PPARα antagonist)	30 mg/kg (PEA) and 2 mg/kg (GW6471)	intraperitoneal, intraplantar, intratechal and i.c.v.	ICR Mice	PEA treatment had antiallodynic effects and the treatment with GW6471 (PPARa antagonist) reversed these effets	Donvito et al, 2016
Neuropathic and Inflammatory	Chronic constriction injury of sciatic nerve (CCI), Freund's adjuvant and carrageenean	PEA and GW7647 (PPARα agonist)	PEA (50 mg) and GW7647 (50 mg); PEA (20 mg/kg) s.c	intraplantar and subcutaneos	Swiss Mice	PEA reduced formalin-induced pain at i.pl. doses; GW7647 and PEA reduced hyperalgesic responses in the chronic constriction injury model of neuropathic pain; acute administration of GW7647 and PEA reduced hyperalgesic responses in the complete Freund's adjuvant and carrageenan models of inflammatory pain.	LoVerme et al, 2006

Inflammatory	Formalin	GW7647 (PPARα agonist)	0.1 - 10 μg/10 μl	intraplantar	Swiss Mice	GW7647 has inhibitor phase I and reduced phase II pain behaviour in the formalin-induced inflammatory model. The antinociceptive effects of PPAR-α receptor agonists are blocked by the large conductance potassium channel.	Russo et al, 2007
Inflammatory	Formalin and carrageenean	PEA	0.01 - 50 μg/200 μl	intraperitoneal	Swiss Mice	Reduction in both early and late phases of formalin-induced nociception by PEA at 5 and 50 μg/paw. PPAR-α knockout animals failed to respond to PEA compared to wild-type animals. The injection of carrageenean resulted in a significant reduction of mechanical and thermal threshold values. Both hyperalgesic parameters were strongly reduced by PEA (50 μg)	Sasso et al, 2012
Neuropathic	Chronic constriction injury of sciatic nerve (CCI)	PEA	30 mg/kg/day	subcutaneos (daily)	Wild-type and PPAR-α−/− (KO) C57BL6 mice	On the day 14, PEA prevented pain threshold alterations in Randall-Selitto and Dynamic Plantar Aesthesiometer tests. In PPAR-α null mice PEA treatment failed to induce pain relief	DiCesare Mannelli et al, 2013
Inflammatory	Formalin	GW7647 (PPARα agonist) and GW6471 (PPARα antagonist)	10 μg (GW7647); 10 μg (GW6471)	Intra-mPFC	Sprague-Dawley Rats	Intra-mPFC administration of GW6471, but not GW7647, resulted in delayed onset of the early second phase of formalin-evoked nociceptive behaviour. formalin-evoked nociceptive behaviour was associated with significant reductions in mPFC levels of endogenous PPARα ligands (PEA and OEA)	Okine et al, 2013
Inflammatory	Formalin	GW6471 (PPARα antagonist) and GW9662 (PPARγ antagonist)	3nmol - 5 μl (GW6471); 36 nmol - 5μl (GW9662)	Intra-ACC	Sprague-Dawley Rats	Both antagonists significantly reduced formalin-evoked nociceptive behaviour, suggesting facilitatory/permissive roles for these receptors in the ACC in inflammatory pain	Okine et al, 2016.1
Inflammatory	Formalin	GW9662 (PPARγ antagonist)	14.4nmols/0.2μL	Intra-lPAG	Sprague-Dawley Rats and Wistar Kyoto rats	Pharmacological blockade of PPARγ in the lPAG enhanced formalin-evoked nociceptive behaviour in WKY, but not SD, rats.	Okine et al, 2016.2

Neuropathic	Spared Nerve Injury	PEA and OEA	10 mg/kg/day during 15 days (OEA and PEA) and 6 nmol/mouse (PEA and OEA)	intraperitoneal and intra-mPFC	CD-1 mice	Repeated PEA and OEA treatmentS significantly increased both the thermal and mechanical thresholds in SNI mice. PEA microinjection decreased mechanical threshold with maximum effect at 75 min post-drug. OEA microinjections immediately and transiently reduced mechanical allodynia which lasted up to 30 min post injection	Guida et al, 2015
Inflammatory	Carrageenean	EPT4900 (NAA inhibitor)	10 mg/kg, 19 mg/kg and 25 mg/kg	intraperitoneal	Sprague-Dawley Rats	EPT4900 selectively increased the levels of PEA and inhibited inflammation as well as hyperalgesia in rats treated with an intraplantar injection of carrageenan.	Petrosino et al, 2015
Neuropathic	Oxaliplatin-induced neuropathy	PEA	30 mg/kg (acute and chronic administration)	intraperitoneal	Sprague-Dawley Rats	Single administration of PEA was able to reduce oxaliplatin-dependent pain induced by mechanical and thermal stimuli. The repeated treatment with PEA prevented lowering of pain threshold as well as increased pain on suprathreshold stimulation.	DiCesare Mannelli et al, 2015
Neuropathic	Spinal Cord Injury	Pioglitazone	0,5, 1,5 or 3,0 mg/kg (Pioglitazone) and 2mg/kg (GW9662)	Intraperitoneal	Sprague-Dawley Rats	Pioglitazone treatment significantly increased thermal threshold in spinal cord injured rats compared to the vehicle group. The administration of pioglitazone + GW9662 (PPARγ antagonist) or GW9662 alone did not result in significant differences to post-SCI surgery rats trated with vehicle.	Park et al, 2006
Inflammatory	Formalin and Carrageenean	Fenofibrate and pioglitazone	100 or 300 mg/kg (Fenofibrate; acute and chronic -7 days); 25, 50 or 100 mg/kg (acute pioglitazone)	*Per os* (p.o.) - Chronic treatment of fenofibrate Intraperitoneal - Acute treatment of piogitazone	Swiss Mice and Wistar rats	Chronic and acute administration of fenofibrate and acute administration of pioglotazone did not inhibit nociceptive responses of mice in the hot plate or in the first phase of the formalin test. The chronic treatment with fenofibrate and acute administration of pioglitazone (same doses) attenuated the second phase of the formalin-induced nociceptive response. The prolonged treatment with fenofibrate also attenuated the initial phase of the carrageenean-induced nociceptive behaviour in rats.	Oliveira et al, 2006
Neuropathic	Diabetes-induced neuropathic pain	Pioglitazone	10 mg/kg	Oral (chronic administration - 28 days)	Sprague-Dawley Rats	The chronic administration of pioglitazone did not attenuate the hyperalgesia induced by the high fat diet/streptozotocin(HFD/STZ) model of diabetes.	Byrne et al, 2014

Other	-	Pioglitazone and GW9662 (PPARγ antagonist)	10 or 30 mg/kg (Pioglitazone) 2.5 or 5 mg/kg (GW9662)	Oral (via gavage) - Daily (concomitant with morphine administration or for the prior 2 days before testing)	C57 mice	Treatment with pioglitazone attenuated the development of morphine tolerance. Pioglitazone administration in mice which were not chronically treated with morphine does not have an effect in nociception. Pre-treatment with GW9662 reversed the effects of pioglitazone in morphine-trrated rats. GW9662 alone does not have an effect in nociceptive responses. The development of tolerance for morphine is more pronounced in PPARγ knockout mice.	De Guglielmo et al, 2014
Neuropathic	Sciatic Nerve Ligation (SNL)	Pioglitazone	4.5 and 9.0 mg/mg	Intraperitoneal	Wistar Rats	Both doses of pioglitazone attenuated hyperalgesia in the hot plate test and the cold allodynia effect of rats submitted to SNL.	Garg et al, 2017
Other	-	Pioglitazone and GW9662	20 or 40 mg/kg (pioglitazone) 2 mg/kg (GW9662)	*Per os* (p.o.) (pioglitazone) - daily for 17 days concomitant with morphine treatment Subcutaneos (GW9662)	Wistar Rats	Treatment with pioglitazone attenuated the development of morphine tolerance. GW-9662 administration 30 min before pioglitazone antagonised the mentioned pioglitazone-induced effects.	Ghavimi et al, 2015
Other	-	Pioglitazone and GW9662	5, 10, 20, or 40 mg/kg (pioglitazone) 2 mg/kg (GW9662)	*Per os* (p.o.) (pioglitazone) - daily for 17 days concomitant with morphine treatment Subcutaneos (GW9662) - daily before pioglitazone administration	Wistar Rats	The highest dose of pioglitazone per se did not alter the pain threshold in tail-flick test. Treatment with pioglitazone attenuated the development of morphine tolerance and GW-9662 administration 30 min before pioglitazone antagonised the pioglitazone-induced effects.	Ghavimi et al, 2014

Inflammatory	Complete Freund's adjuvant (CFA)-induced inflammation	Rosiglitazone	0.3, 3 or 30 μg	Intraplantar	C57BL6 Mice	Hyperalgesia to mechanical stimuli was dose-dependently attenuated on days 5 and 7 after the procedure in mice that received rosiglitazone, which was reversed to the level of vehicle-injected mice by coadministration of GW9662. In contrast to the effects of rosiglitazone on mechanical stimuli, rosiglitazone had little effect on withdrawal latency to heat stimuli.	Hasegawa-Moriyama et al, 2013
Neuropathic	Partial Sciatic Nerve Ligation (PSL)	Pioglitazone	1, 5 or 25 mg/kg	*Per os* (p.o.) during 5 days	ICR Mice	Pioglitazone reduced the tactile allodynia at all doses. However, pioglitazone did not affect nociceptive responses in sham mice.	Iwai et al, 2008
Neuropathic	Tibial and sural nerve transection (TSNT)	Rosiglitazone	2.5, 5 or 10 mg/kg	*Per os* (p.o.) daily for 28 days	Wistar Rats	Administration of rosiglitazone (at 5 and 10mg/kg) reduced the mechanical and cold hyperalgesia induced by TSNT without affecting heat hyperalgesia.	Jain et al, 2009
Neuropathic	Lumbar 5 spinal nerve transection	Pioglitazone	2.5, 5 or 10 mg/kg	*Per os* (p.o.) daily for 14 days	Sprague-Dawley Rats	Pioglitazone (5 and 10 mg/kg) attenuated mechanical hyperalgesia produced by lumbar 5 spinal nerve transection	Jia et al, 2010
Neuropathic	Trigeminal inflammatory Compression (TIC)	Pioglitazone, GW0742 (PPAR β/δ agonist), Bezafibrate, Fenofibrate, GW9662	100, 300 or 600 mg/kg (pioglitazone); 1 or 6 mg/kg (GW0742); 100 mg/kg (Bezafibrate); 200 mg/kg (Fenofibrate); 30 mg/kg (GW9662)	Oral - pioglitazone 600 mg/kg and bezafibrate 100 mg/kg Intraperitoneal - all the others	C57BL/6 mice	Systemic administration of pioglitazone attenuates whisker pad mechanical allodynia at doses of 300 mg/kg and 600 mg/kg. Administration of GW9662 prior to pioglitazone (300 mg/kg) blocked the analgesic effect of pioglitazone. GW0742 (6 mg/kg) partially attenuated mechanical allodynia in mice with TIC injury compared to vehicle treated mice.	Lyons et al, 2017
Inflammatory	Formalin	GW9662 and Pioglitazone	2 mg/kg (GW9662) and 10, 20, 30 or 50 mg/kg (pioglitazone)	Intraperitoneal	Wistar Rats	Pioglitazone at doses 30 and 50 mg/kg significantly inhibited the flinching behaviour in phase 1 and, at dose 30 mg/kg, in phase 2. GW9662 had no effect in nociceptive behaviour per se, but it attenuted antinociceptive effects of the combined treatment of simvastatin and pioglitazone.	Mansouri et al, 2017

Inflammatory	Formalin	GW9662 and Pioglitazone	2 mg/kg (GW9662) and 10, 20, 30 or 50 mg/kg (pioglitazone)	Intraperitoneal and intraplantar	Wistar Rats	Both routes of pioglitazone administration produced antinociception in both phases of formalin-induced pain. Antinociception caused by i.p. and i.pl. pioglitazone was blocked by GW-9662 at doses 2 mg/kg (i.p.) and 3 μg/paw (i.pl.).	Mansouri et al, 2017
Neuropathic	Chronic constriction injury (CCI)	Pioglitazone	20 mg/kg/day	Oral - daily for 14 days	Sprague-Dawley Rats	Pioglitazone attenuated the CCI-induced mechanical and thermal hyperalgesia.	Murad et al, 2015
Visceral	Diarrhoea-predominant Irritable Bowel Syndrome (D-IBS)	Pioglitazone and GW9662	2 mg/kg (pioglitazone); 3mg/kg (GW9662)	Intraperitoneal on days 7,9 and 11.	Wistar Rats	Pioglitazone reduced visceral hypersensitivity and defe- cation frequency and increased nociceptive thresholds.	Paragomi et al, 2014
Neuropathic	Spinal Nerve Ligation (SNL)	Pioglitazone	5, 10 or 20mg/kg	Intraperitoneal - dialy for 28 days	Sprague-Dawley Rats	Higher doses of pioglitazone attenuated the SNL-induced mechanical allodynia, cold allodynia, mechanical hyperalgesia and thermal hyperalgesia.	Pottabathini et al, 2015
Other	Incisional pain	Rosiglitazone	25 μg	Intraplantar (in loci)	BKS.Cg-+Leprdb/+Leprdb/Jc l Mice	Rosigitazone alleviates mechanical hyperalgesia resulted by the incision.	Saito et al, 2015
Inflammatory	Complete Freund's adjuvant (CFA)-induced	-	-	-	Wistar Rats	PPARa was rapidly activated in lumbar spinal cord after CFA intraplantar injection.	Benani et al, 2004
Inflammatory	Carrageenean	PEA and siRNA for PPARα	1.0 μg/h	i.c.v.	Sprague-Dawley Rats	Lower PPARα expression was observed in the spinal cord of High Fat-fed rats. PEA significantly attenuated thermal and mechanical hyperalgesia in HF-fed rats. Intrathecal administration of PPARα siRNA completely abolished the effects of ICV PEA on pain sensitivity.	Wang et al, 2014
Inflammatory	Carrageenean (Diet-induced obesity)	siRNA for PPARα	10 μl	Intrathecal	Sprague-Dawley Rats	Knockdown of spinal PPARα eradicated the beneficial effects of Ursolic Acid on thermal hyperalgesia and paw edema, and reversed the spinal cord inflammatory response.	Zhang et al, 2016

Inflammatory	Formalin, Carrageenean and Writhing test	Oleanoic Acid (OA)	10, 20 and 40 mg/kg	Oral	Swiss Mice	OA treatment inhibits acetic acid-induced abdominal writhes in mice. OA alone did not produce a significant effect on the first phase of the formalin test but reduced the number of paw licks in the second phase of the formalin test.	Vasconcelos et al, 2006
Neuropathic	Oxaliplatin-induced neuropathic pain	15 Thiazolidinones (TZDs) and GW9662	40 mg/kg (TZDs) 4 mg/kg (GW9662)	Intraperitoneal	C57BL/6 mice	Except for compound 14, all TZDs showed antinociceptive properties; these TZDs attenuated Oxaliplatin-induced mechanical hyperalgesia. This effect was prevented by GW9662 (PPARg antagonist).	Moreira et al, 2017
Inflammatory	Formalin	PEA and GW6471	0.2 and 2 mg/kg (GW6471i.p.), 0.2 or 1 µg/5µL/mouse (GW6471 i.t.), 1 µg/20µL/mouse (GW6471 i.pl.) 1 or 3 mg/kg (PEA I.p.)	Intraperitoneal (GW6471 was also administered intraplantar and intrathecal)	ICR Mice and -/-α7 Mice (C57BL/6 background)	GW6471 blocks and PEA potentiates the antinociceptive effects of α7 nAChR full agonist. PEA and GW6471 alone do not affect formalin-evoked nociceptive responses.	Donvito et al, 2017
Inflammatory and Neuropathic	Chronic Constriction Injury (CCI) and Acetic acid-, magnesium sulfate- and kaolin-evoked writhing and Formalin	GW9662 and PEA	PEA (20 mg/kg/ip) GW9662 (2 mg/kg)	Intraperitoneal	Swiss CD1 Mice and PPARa null type Mice	PEA had antihyperalgesic effect on mechanical and thermal stimulus. Single intraperitoneal administration of GW9662 produced a reversion of analgesic effect of both compounds tested (butyrate and FBA).	Russo et al, 2015
Neuropathic	Sciatic Nerve Ligation	GW6471	5, 10 or 20 mg/kg	Intraperitoneal	C57BL/6 wild-type and PPARα null lineages	higher sensitivity to thermal and mechanical non-noxious and noxious stimuli, and cold and mechanical allodynia and heat hyperalgesia was observed in mice lacking PPAR-α. Writhes after acetic acid were also enhanced in mutant mice. The blockade of PPARa did not alter nociceptive behaviour.	Ruiz-Medina et al, 2012
Other	Incisional pain	Rosiglitazone	0.5 mg/ml	Local (intraplantar)	C57BL/6 Mice	Local administration of rosiglitazone immediately after the procedure ameliorates thermal and mechanical hyperalgesia.	Hasegawa-Moriyama et al, 2012

Neuropathic	Oxaliplatin-induced neuropathic pain	Rosiglitazone	3 and 10 mg/kg	*Per os* (p.o.) daily for 20 days	Sprague-Dawley Rats	Rosiglotazine attenuated hyperalgesia and allodynia resulted by Oxaliplatin neuropathy.	Zanardelli et al, 2014
Neuropathic	Trigeminal nerve injury (TNI)	Pioglitazone	100 mg/kg	Intraperitoneally- daily for 7 days	C57BL/6 Mice	DCS (NMDA agonist) and pioglitazone combination n attenuated orofacial neuropathic pain and anxiety related behaviours. The treatment with pioglitazone alone did not alter nociceptive behaviour.	Lyons et al, 2017
Neuropathic and Visceral	Acetic Acid induced visceral pain, scietic nerve injury (SNI)	MK886 (PPARα antagonist)	2mg/kg	Intraperitoneal	ICR mice, Kunming mice and C57BL/6J mice and PPAR-α knockout mice (-/-)	F96 (selective NAAA inhibitor) had an overall anti-nociceptive effect in the different models and tests carried out in the study. This effect was widely blocked by PPARα antagonist MK886 and by genetic disruption of PPAR-α	Yang et al, 2015
Inflammatory	Formalin - Tempomandibular joint (TMJ)	15d-PGJ2 and GW9662	0.3, 1 or 3 ng/15 μl/TMJ (GW9662) 100 ng/15 μl/TMJ (15d-PGJ2)	Intra-TMJ	Wistar Rats	Treatment with 15d-PGJ2 attenuated formalin-evoked nociceptive behaviour in the TMJ. This effect was blocked by GW9662 (PPARγ antagonist)	Pena-dos-Santos et al, 2009
Inflammatory	Carrageenean	URB597, GW6471 and WY14643 (PPARα agonist)	25 μg in 50μL (URB597) 30 μg in 50μL (GW6471) 100 μg in 50μL (WY14643)	Intraplantar	Sprague-Dawley Rats	GW6471 completely abolished the inhibitory effects of URB597 on the carrageenan-evoked expansion of receptive fields (8 g) and WY14643 significantly attenuated carrageenan-evoked expansion of peripheral receptive fields of WDR neurons.	Sagar et al, 2008
Inflammatory	Carrageenean and Formalin - Tempomandibular joint (TMJ)	15d-PGJ2 and GW9662	30–300 ng/paw (15d-PGJ2)	Intraplantar or Intra-TMJ	Wistar Rats	15d-PGJ2 inhibits the mechanical hypernociception induced carrageenan in the hindpaw and formalin in the TMJ These effects were blocked by GW9662.	Napimoga et al, 2007

Visceral and Inflammatory	Formalin and Writhing	OEA WY14643	0.5, 1, 5, 10 and 20 mg/kg (OEA) 5 and 20 mg/kg (WY14643)	Intraperitoneal	CD1 Mice (wild type and PPARα null)	Treatment with OEA decreased the writhing response induced by acetic acid and formalin-evoked nociceptive behaviour (both phases) in both wild and KO mice. WY14643 did not affect the early phase of the formalin test whereas it slightly decreased the late phase	Suardíaz et al, 2007
Neuropathic	Peripheral Nerve Injury - L5 Spinal Nerve Transection	Pioglitazone and GW9662	10 mg/kg (Pioglitazone) 2 mg/kg (GW9662)	Intraperitoneal	Sprague-Dawley Rats	Pioglitazone improved the mechanical hyperalgesia in operated rats. This effect was reversed by GW9662	Jia et al, 2013

Appendix B

Buffers and solution for Western Blotting

4X Sample Buffer: stored @ -20°C

Ingredients:	20 ml:
• SDS	1 g
• 1 M Tris-HCl pH 6.8	5 ml
• Glycerol	4 ml
• 1% Bromophenol Blue (PBS or PMF)	500 ul
• dH_2O	make it up to 20ml
• Make 800µl aliquots	

Add 200µl 2-mercaptoethanol to aliquot when ready to use

First add the bromophenol blue to the 5ml of tris-HCl. After stirring it well, add SDS until is dissolved (to dissolve it add to maximum 5ml of water). Then add the glycerol and finally make it up to 20 ml.

RIPA Lysis Buffer: stored @ 4°C

The lysis buffer is made up from several other buffers/solutions, therefore these need to be made first;

(i) 50mM Ethylene Glycol Tetraacetic Acid (EGTA) Solution

To make up 10ml solution;

- 0.19g of EGTA
- Add 10ml of dH_2O and dissolve by shaking and vortexing.
- Store @ room temperature

(ii) 50mM Sodium Fluoride (NaF) Solution

To make up 100ml solution;

- 0.21g of NaF

- Add 100ml of dH_2O and dissolve by inverting.
- Store @ room temperature

(iii) 1M Tris-Hydrochloric Acid (Tris-HCL) Solution

To make up a 100ml solution;

- 15.15g Trizma Base
- Add 50ml dH_2O and place on stirrer to dissolve
- pH adjusted to 7.4
- Make a final volume of 100ml in dH_2O
- Store @ room temperature

(iv) 5M Sodium Chloride (NaCl) Solution

To make up a 50ml solution;

- 14.63g of NaCl
- Add 50ml dH_2O and vortex to dissolve.
- Store @ room temperature

(v) 10% Sodium Deoxycholate

To make up a 1ml solution;

- 0.1g sodium deoxycholate
- Transfer into 1.5ml Eppendorf using 1ml of dH_2O, vortex to dissolve.

(vi) 1mM Sodium orthovanadate

To make up a 50ml solution;

- 0.01g sodium orthovanadate
- Transfer into 50ml Eppendorf using a few mls of dH_2O, bring up to 50ml and vortex to dissolve.

Using these prepared buffers make up the Lysis Buffer as follows;

Lysis Buffer Preparation

- 50mM EGTA (240µL)
- 50mM NaF (120µL)

- 1M Tris-HCL (600µL)
- 5M NaCl (360µL)
- 0.25% of sodium deoxycholate (300µL of a 10% sodium deoxycholate solution)
- 1mM sodium orthovanadate (120µL)
- NP-40/Igepal CA-630 (120µL) **(*Available pre-made on shelf)**

- Dissolve to a final volume of 12mls in dH_2O
- Aliquot 990µl into twelve 1.5ml Eppendorf microfuge tubes
- Stored @ -20°C

Immediately before use, **10µl of protease inhibitor cocktail** (Sigma, P8340) was added to each Eppendorf microfuge tube of lysis buffer as the stability of this solution is undetermined.

4x Separation Gel buffer: stored @ room temperature

Ingredients:	100 ml:
• Trizma-base	18.2 g
• 0.4% SDS	4ml 10%
• pH 8.8 with conc HCL	
• then make it upto 100 ml volume	

4x Stacking Gel buffer: stored @ room temp

Ingredients:	100 ml:
• Trizma-base	6 g
• 0.4% SDS	4ml 10%
• pH 6.8 with conc HCL	
• then make it upto 100 ml volume	

4x Separation Gel buffer: stored @ room temp

Ingredients:	100 ml:
• Trizma-base	18.2 g
• 0.4% SDS	4ml 10%

- pH 8.8 with conc HCL
- then make it upto 100 ml volume

4x Stacking Gel buffer: stored @ room temp

Ingredients:	100 ml:
• Trizma-base	6 g
• 0.4% SDS	4ml 10%

- pH 6.8 with conc HCL
- then make it upto 100 ml volume

10% Ammonium persulfate (100 mg/ml) Make fresh weekly and store @ 4°C

Required while making up gels eg: 0.05g in 500ul

Running buffer 10x: stored @ room temperature

Ingredients:	1L(10x)
• Trizma-base (25 mM)	30 g
• Glycine (192 mM)	144 g
• SDS (10%)	100ml
• dH_2O	1L

NOTE: dilute it to 1x before use

Transfer buffer (10x): stored @ room temp

Ingredients:	1L:
• Trizma-base (25 mM)	30g
• Glycine (192 mM)	144 g
• dH_2O	make it upto 1000ml

Transfer buffer (1X): stored @ 4°C

Actually required up to 1.5L and used in chilled condition

Ingredients:	1.5L:
• Transfer buffer(10x)	150ml
• Methanol	300ml
• Water	make it upto 1500ml

10. 10X Tris-Buffered Saline (TBS) stored @ 4°C

Ingredients:	1000ml:
• 200 mM Trizma-base	24.23 g
• 1.37 M NaCl	80.06 g
• dH_2O (dissolve in on stirrer)	800ml
• pH to 7.6	
• dH_2O (add after adjusting pH)	200ml

Blocking solution: stored @ 4°C

Ingredients:	50 ml:
• Milk powder (5% milk)	2.5g
• TBSTween solution (0.1%)	make it upto 50 ml

Washing solution (0.1%TBST) stored room temp

Ingredients:	1000ml:
• Tween 20 (detergent)	500ul
• 1X TBS	1000ml

Primary Antibody diluent: stored @ 4°C

- 5 ml blocking solution (5 % milk)
- Calculate the required primary antibody concentration
- Generally 1 in 10,000 concentration of Beta-actin (house) is prepared in 5% milk (blocking solution) and to it the target (the receptor what we are looking for?)

Secondary Antibody diluent: stored @ 4°C

- To the 1% milk solution(0.5g in 50ml of TBST)
- add 5ul of each secondary antibodies

Separation gel (for 2 gels of 10 cm each)

Ingredients:	10%	**8%**	9%
• 30% acryl amide	6.7ml	**5.3ml**	5 ml
• Separation gel buffer(4x)	5ml	**5ml**	**5ml**
• ddH_2O	8.3ml	**9.5ml**	
• TEMED	20ul	**20ul**	**20ul**
• 10% Ammonium Sulphate	200 µl	**200ul**	**200ul**

Add the ingredients from top to bottom and make sure the APS is fresh and added in the last and immediately poured in between plates.

Stacking gel (10ml would be sufficient for 2 gels)

Ingredients:	
• 30% acryl amide	1ml
• Stacking gel buffer(4x)	2.5ml
• ddH_2O	6.5ml
• TEMED	10ul
• 10% Ammonium Sulphate (APS)	100 µl

Add the ingredients from top to bottom and make sure the APS is fresh and added in the last and immediately poured in between plates.

Stripping buffer: stored @ RT

• Glycine	0.375 g

Dissolve the glycine in 100ml of dH_2O. Adjust the pH of the solution to 2.0 by adding HCl. Add 20 ml of 20% SDS solution (see above) and make it to 200ml with dH_2O.

Membrane Stripping – protocol

1. Pour some stripping buffer (±20ml) on blot and place in water bath at 45C for 20 minutes.

2. Pour off the buffer and repeat.
3. Wash blot for three times for 10 minutes with TBST 0.1%
4. Block for 1hr with 5% fat milk solution.
5. Repeat the same WB protocol used before stripping. (i.e. Blocking followed by primary antibody overnight, washes and secondary antibody for 1 hour in the following day).

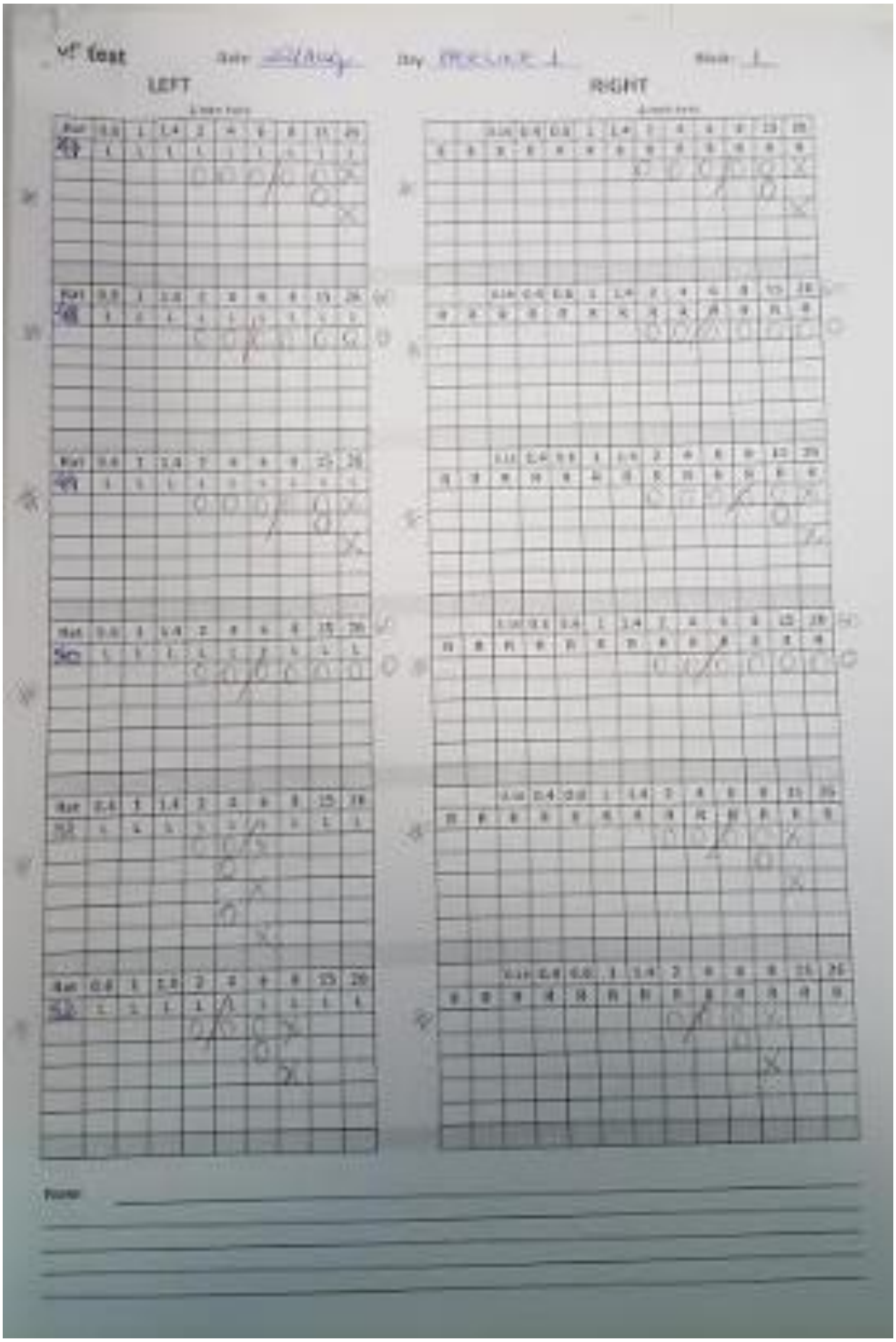
Appendix C

Table C.1: Values of constants (κ) for estimating LD50 from up-and-down method (from *Dixon, 1965 - The up-and-down method for small samples*).

N	Second Part of Series	k for Test Series Whose First Part is					Standard Error of LD_{50}
		O	*OO*	*OOO*	*OOOO*		
2	*X*	−.500	−.388	−.378	−.377	*O*	.88σ
3	*XO*	.842	.890	.894	.894	*OX*	.76σ
	XX	−.178	.000	.026	.028	*OO*	
4	*XOO*	.299	.314	.315	.315	*OXX*	.67σ
	XOX	−.500	−.439	−.432	−.432	*OXO*	
	XXO	1.000	1.122	1.139	1.140	*OOX*	
	XXX	.194	.449	.500	.506	*OOO*	
5	*XOOO*	−.157	−.154	−.154	−.154	*OXXX*	.61σ
	XOOX	−.878	−.861	−.860	−.860	*OXXO*	
	XOXO	.701	.737	.741	.741	*OXOX*	
	XOXX	.084	.169	.181	.182	*OXOO*	
	XXOO	.305	.372	.380	.381	*OOXX*	
	XXOX	−.305	−.169	−.144	−.142	*OOXO*	
	XXXO	1.288	1.500	1.544	1.549	*OOOX*	
	XXXX	.555	.897	.985	1.000^{+1}	*OOOO*	
6	*XOOOO*	−.547	−.547	−.547	−.547	*OXXXX*	.56σ
	XOOOX	−1.250	−1.247	−1.246	−1.246	*OXXXO*	
	XOOXO	.372	.380	.381	.381	*OXXOX*	
	XOOXX	−.169	−.144	−.142	−.142	*OXXOO*	
	XOXOO	.022	.039	.040	.040	*OXOXX*	
	XOXOX	−.500	−.458	−.453	−.453	*OXOXO*	
	XOXXO	1.169	1.237	1.247	1.248	*OXOOX*	
	XOXXX	.611	.732	.756	.758	*OXOOO*	
	XXOOO	−.296	−.266	−.263	−.263	*OOXXX*	
	XXOOX	−.831	−.763	−.753	−.752	*OOXXO*	
	XXOXO	.831	.935	.952	.954	*OOXOX*	
	XXOXX	.296	.463	.500	$.504^{+1}$	*OOXOO*	
	XXXOO	.500	.648	.678	.681	*OOOXX*	
	XXXOX	−.043	.187	.244	$.252^{+1}$	*OOOXO*	
	XXXXO	1.603	1.917	2.000	2.014^{+1}	*OOOOX*	
	XXXXX	.893	1.329	1.465	1.496^{+1}	*OOOOO*	
		X	*XX*	*XXX*	*XXXX*	Second Part of Series	
		$-k$ for Series Whose First Part is					

Appendix D

Example of the test table for the up-and-down method of assessing mechanical allodynia measured by von Frey testing



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