

Genotype-dependent responsiveness to inflammatory pain: The role of TRPV1 in the periaqueductal grey

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Abstract

The ability to experience pain is essential for survival, and to prevent potential tissue damage upon exposure to noxious stimuli. The periaqueductal grey - rostral ventromedial medulla – dorsal horn of the spinal cord (PAG-RVM-DH) pathway plays a pivotal role in pain processing and modulation. Antinociception caused by activation of this descending inhibitory pain pathway involves the PAG-mediated activation of neurons within the RVM. Negative affective state has a significant impact on pain, and genetic background is an important moderating influence on this interaction. The Wistar–Kyoto (WKY) inbred rat strain exhibits a stress-hyperresponsive, anxiety/depressive-like phenotype and also displays a hyperalgesic response to noxious stimuli, compared with Sprague-Dawley (SD) rats. Transient receptor potential subfamily V member 1 (TRPV1), a non-selective ligand-gated ion-channel activated by protons, capsaicin (active constituent of chilli peppers), and thermal stimuli, plays a key role within the midbrain PAG in regulating both aversive and nociceptive behaviour. The overall aim of the studies described in this thesis was to investigate the role of TRPV1 in the columns of the PAG in hyperalgesia to formalin-evoked inflammatory pain in WKY versus SD rats. TRPV1 mRNA expression was significantly lower in the dorsolateral (DL) PAG and higher in the lateral (L) PAG of WKY rats, compared with SD counterparts. There were no significant differences in TRPV1 mRNA expression in the ventrolateral (VL) PAG between the two strains. TRPV1 mRNA expression significantly decreased in the DLPAG and increased in the VLPAG of SD, but not WKY rats, upon intra-plantar formalin administration. Intra-DLPAG administration of either the TRPV1 agonist capsaicin, or the TRPV1 antagonist 5'-Iodoresiniferatoxin (5'-IRTX), significantly increased formalin-evoked nociceptive behaviour in SD rats, but not in WKY rats. The effects of capsaicin were likely due to TRPV1 desensitisation, given their similarity to the effects of 5'-IRTX. Intra-VLPAG administration of capsaicin or 5'-IRTX reduced nociceptive behaviour in a moderate and transient manner in SD rats, and similar effects were seen with 5'-IRTX in WKY rats. Intra-LPAG administration of 5'-IRTX reduced nociceptive behaviour in a moderate and transient manner in SD rats, but not in WKY rats. Pharmacological modulation of TRPV1 in the columns of the PAG had little effect on the levels of neurotransmitters and endocannabinoids/N-acylethanolamines in the RVM and dorsal horn of the spinal cord or on *c-Fos* expression in the dorsal horn. These results indicate that modulation of inflammatory pain by TRPV1 in the PAG occurs in a column-

specific manner. The data also provide evidence for differences in the expression of TRPV1, and differences in the effects of pharmacological modulation of TRPV1 in specific PAG columns, between WKY and SD rats, suggesting that TRPV1 expression and/or functionality in the PAG plays a role in hyper-responsivity to noxious stimuli in a genetic background prone to negative affect.

Author's Declaration

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

1. Aspects of the TRPV1 mRNA analysis study, including tissue generation and harvesting, mRNA isolation and cDNA synthesis were carried by Dr. Weredeselam Olango
2. Some of the early intracerebral cannula implantation surgeries were performed by Dr. Kieran Rea and Dr. Bright Okine who assisted in my training in this technique

The thesis or any part thereof has not been submitted to any other institution in connection with any other academic award. Any views expressed herein are those of the author.

Signed: _____

Date: _____

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Abbreviations

2-AG: 2-arachidonylglycerol

5'-IRTX: 5'-Iodoresiniferatoxin

5-HT: 5-hydroxytryptamine

ACC: Anterior cingulate cortex

AEA: Anandamide

AMPA: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA: Analysis of variance

BDNF: Brain-derived neurotropic factor

CAP: Capsaicin

CAP+5'-IRTX: Capsaicin in combination with 5'-Iodoresiniferatoxin

CB₁: Cannabinoid receptor type 1

CB₂: Cannabinoid receptor type 2

CCI: Chronic constriction injury

CCK: Cholecystokinin

CCK-₂: Cholecystokinin 2 receptor

CeA: Central nucleus of the amygdala

CFA: Complete Freund's adjuvant

CMS: Chronic mild stress

CRF₁: Corticotropin-releasing hormone receptor

DAGL: Diacylglycerol lipase

DLPAG: Dorsolateral periaqueductal grey

DMH: Dorsomedial hypothalamus

DMSO: Dimethylsulfoxide

DPAG: Dorsal periaqueductal grey

DR: Dorsal raphe

DRG: Dorsal root ganglia

FAAH: Fatty acid amide hydrolase

FCA: Fear-conditioned analgesia

FST: Forced swim test

GABA: Gamma-aminobutyric acid

GFAP: Glial fibrillary acidic protein

HPA: Hypothalamo-pituitary-adrenal

IASP: International Association for the Study of Pain

IBS: Irritable bowel syndrome

LC: Locus coeruleus

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

LPAG: Lateral periaqueductal grey

MAGL: Monoacylglycerol lipase

MD: Maternal deprivation

mGLu: Metabotropic glutamate receptor

mRNA: Messenger ribonucleic acid

MS: Maternal separation

NA: Noradrenaline

NAPE: N-arachidonoyl-phosphatidylethanolamine

NAT: *N*-acyltransferase

NMDA, N-methyl-D-aspartic acid

NOP: nociceptin/orphanin FQ peptide

OEA: Oleoylethanolamine

PAG: Periaqueductal grey

PBS: Phosphate buffered saline

PE: Phosphatidylethanolamine

PEA: Palmitoylethanolamide

PEAP: Place escape avoidance paradigm

PFA: Paraformaldehyde

PFC: Prefrontal cortex

PLC: Phospholipase C

PND: Post-natal day

PPAR α : Peroxisome proliferator-activated receptor alpha

PTSD: Post-traumatic stress disorder

RVM: Rostral ventromedial medulla

S1: Somatosensory cortex

SART: Specific alternation rhythm of temperature

SD: Sprague-Dawley

SIH: Stress-induced hyperalgesia

SNL: Spinal nerve ligation

SSRI: Selective serotonin re-uptake inhibitor

TMJ: Temporomandibular joint

VLPAG: Ventrolateral Periaqueductal grey

WAS: Water avoidance stress

WKY: Wistar-Kyoto

List of Publications and Presentations Arising from the Work Presented in this Thesis

Appendix:

Original research articles (peer reviewed)

Madasu MK, Okine BN, Rea K, Olango WM, Lenihan R, Roche M, Finn DP (2016). Genotype-dependent responsivity to inflammatory pain: a role for TRPV1 in the periaqueductal grey. *Pharmacological Research*. 2016;113(Pt A):44-54.

Okine BN, Madasu MK, McGowan F, Prendergast C, Gaspar JC, Harhen B, Roche M, Finn DP N-palmitoylethanolamide in the anterior cingulate cortex attenuates inflammatory pain behaviour indirectly via a CB1 receptor-mediated mechanism. (Accepted for publication in *PAIN*)

Okine BN, Gaspar JC, Madasu MK, Olango WM, Harhen B, Roche M, Finn DP. Neurochemical, molecular and pharmacological characterisation of peroxisome proliferator activated receptor signalling in the rat midbrain periaqueductal grey in a genetic background prone to heightened stress, negative affect and hyperalgesia. (Under revision for publication in *Brain Research*)

Okine BN, Rea K, Olango WM, Price J, Madasu MK, Roche M, Finn DP (2014). A role for PPAR- α in the medial prefrontal cortex in formalin-evoked nociceptive responding in rats. *British Journal of Pharmacology*, 171(6):1462-1471.

Rea K, Olango WM, Okine BN, Madasu MK, Coyle K, Harhen B, Roche M, Finn DP (2014). Impaired endocannabinoid signalling in the rostroventromedial medulla underpins genotype-dependent hyper-responsivity to noxious stimuli. *PAIN*, 155(1): 69-79.

Review (peer-reviewed):

Madasu MK, Roche M and Finn DP. Spinal TRPV1 in Pain and Psychiatric Disorders. In: *Pain in Psychiatric Disorders -A volume in the series 'Modern Trends in Pharmacopsychiatry*. Basel, Karger, 2015, vol 30, pp 80–93 (DOI:10.1159/ 000435934)

Abstracts (peer reviewed/published):

Madasu MK, Okine BN, Olango WM, Roche M and Finn DP (2014): Differential effects of pharmacological modulation of TRPV1 in the lateral periaqueductal grey on formalin-evoked nociceptive behaviour in Sprague-Dawley and Wistar-Kyoto rats. 24th Annual Symposium of the International Cannabinoid Research Society, Baveno, Italy- Poster presentation

Okine BN, Madasu MK, Gowan F, Harhen B, Roche M, Finn DP (2014). Direct administration of n-palmitoylethanolamide into the rat medial prefrontal cortex reduces formalin-evoked nociceptive behaviour via a CB₁ receptor-mediated mechanism. 24th Annual Symposium on the Cannabinoids, International Cannabinoid Research Society, Baveno, Italy – Poster presentation

Madasu MK, Okine BN, Olango WM, Roche M and Finn DP (2013). TRPV1 mRNA Expression within the CNS of two rat strains differing in nociceptive responsivity. Proceedings of the British Pharmacological Society in 6th Endocannabinoid Conference. Dublin, Ireland-Poster presentation

Okine BN, Madasu MK, Gowan F, Harhen B, Roche M, Finn DP (2014). Intra-mPFC administration of the endogenous PPAR agonist N-palmitoylethanolamide reduces formalin-evoked nociceptive behavior in rats via a CB1 receptor-mediated mechanism 15th World Congress on pain. Bon-aries, Argentina- Poster presentation

Madasu MK, Okine BN, Olango WM, Roche M and Finn DP (2015). TRPV1 in the Dorsolateral Periaqueductal Grey Differentially Modulates Formalin-evoked Nociceptive Behaviour in Sprague-Dawley and Wistar-Kyoto Rats. 7th European Cannabinoid workshop. Sestri Levante, Italy - Poster presentation

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Chapter 1. Introduction

1.1 Introduction

The International Association for the Study of Pain defines pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage’. Pain is a subjective and multidimensional experience that can have a significant impact on the physiological and psychological state of an individual. It is also a major unmet clinical need, posing a major challenge to healthcare professionals. The ability to experience acute pain is essential for survival and prevents potential tissue damage. However, chronic pain has no physiological value and is often debilitating. Chronic pain can be defined as pain persisting for 3–6 months or longer and may be neuropathic, inflammatory or idiopathic in nature (Basbaum et al., 2009). Breivik et al 2006 reported that one in five Europeans suffer from chronic pain. There is an unprecedented clinical need for improved treatment of pain, with patients in primary care settings across the globe reporting the prevalence of persistent pain of 10 to 25% (Breivik et al., 2006; Reid et al., 2011). Chronic pain is the most disabling and costly medical condition, with a direct cost of \$100 billion annually in the US (McCarberg et al., 2006). Symptoms of chronic pain can include hyperalgesia (increased pain from a stimulus that normally provokes pain) and allodynia (pain arising from normally innocuous stimuli). Balance of inhibition/facilitation of pain is maintained throughout life under normal conditions, to ensure only stimuli of a certain threshold are perceived as painful in a manner that is conducive to health and survival. Development of chronic pain is thought to involve descending facilitation and/or peripheral and central sensitization. Inflammatory-mediated changes triggered by tissue damage cause peripheral sensitization, resulting in an increase in responsiveness of nociceptors and reduction in pain threshold (Curatolo et al., 2006). In contrast, central sensitization is an increase in the excitability of neurons within the central nervous system (CNS) especially at the level of the dorsal horn of the spinal cord. Prolonged, intense injury-induced activation of primary afferent neurons (A δ - and C-fibres) generally leads to central sensitization. A reduced activation threshold and an augmented response to stimuli can occur when normally sub-threshold inputs are recruited producing an increased output (Latremoliere and Woolf, 2009). Current pharmacological treatments (e.g. non-steroidal anti-inflammatory drugs [NSAIDs],

opioids, gabapentinoids and anticonvulsants, antidepressants) provide an arsenal of treatments for chronic pain conditions, however these treatment regimens are ineffective in a significant percentage of patients or associated with significant adverse effects.

Clinical studies provide evidence that intensity of pain is influenced by emotionality and there is very high comorbidity of chronic pain with depression and anxiety disorders (Bair et al., 2003; Banks and Kerns, 1996; Magni et al., 1990; McWilliams et al., 2003). Furthermore, it has been hypothesised that negative affect can influence the transition from acute to chronic pain (Linton, 2000). Negative affect is a general dimension of subjective distress and unpleasurable engagement that subsumes a variety of aversive mood states, including anger, contempt, disgust, guilt, fear, and nervousness (Tellegen, 1985; Watson et al., 1988). High negative affective state is a distinguishing feature of anxiety (Tellegen, 1985; Watson and Clark, 1984). The precise relationship between emotionality and pain remains unclear. Previous studies suggest a reciprocal relationship between chronic pain and negative affect and support the concept of a self-perpetuating cycle of events that may underpin the chronic nature of such comorbid disorders (Knaster et al., 2011). Therefore, investigation of the neurobiological mechanisms of pain, and its modulation by negative affect, will inform the identification of new therapeutic targets for improved treatment of pain.

1.2 Pain Pathways

In response to a noxious stimulus, nociceptive information (in the form of action potentials) is relayed via primary afferent neurons to the dorsal horn of the spinal cord (Figure 1.1). Primary afferent neurons are classified on the basis of diameter, structure and conduction velocity into A-delta- and C-fibres. Under normal conditions, A-delta fibres, which are myelinated, elicit a rapid sharp type of pain, whereas C- fibres which are unmyelinated evoke a late, dull pain that lasts longer (Millan, 1999). Substances such as kinins, nitric oxide, histamine, prostanoids, adenosine and serotonin act on the sensory fibres (Braszko and Kościelak, 1975; Fox et al., 1974; Hütter and Strein, 1988; Rosenthal, 1977). Nociceptive information is relayed onto laminae I, II and IV-VIII within the ten laminae of the dorsal horn of spinal cord (Molander and Grant, 1986; Swett and Woolf, 1985). From there, the information is conducted to the brain via the ascending pain pathways by the second order neurons (Willis, 1985a; Willis, 1985b). In

the dorsal horn, the activity of the second order neurons is modulated by neurons of the descending pain pathway which release neurotransmitters resulting in facilitation/inhibition (Basbaum et al., 1978; Dickenson and Le Bars, 1987; Fritschy et al., 1987; Heinricher et al., 2009; Ruda et al., 1981; Sikandar et al., 2012; Rahman et al., 2006).

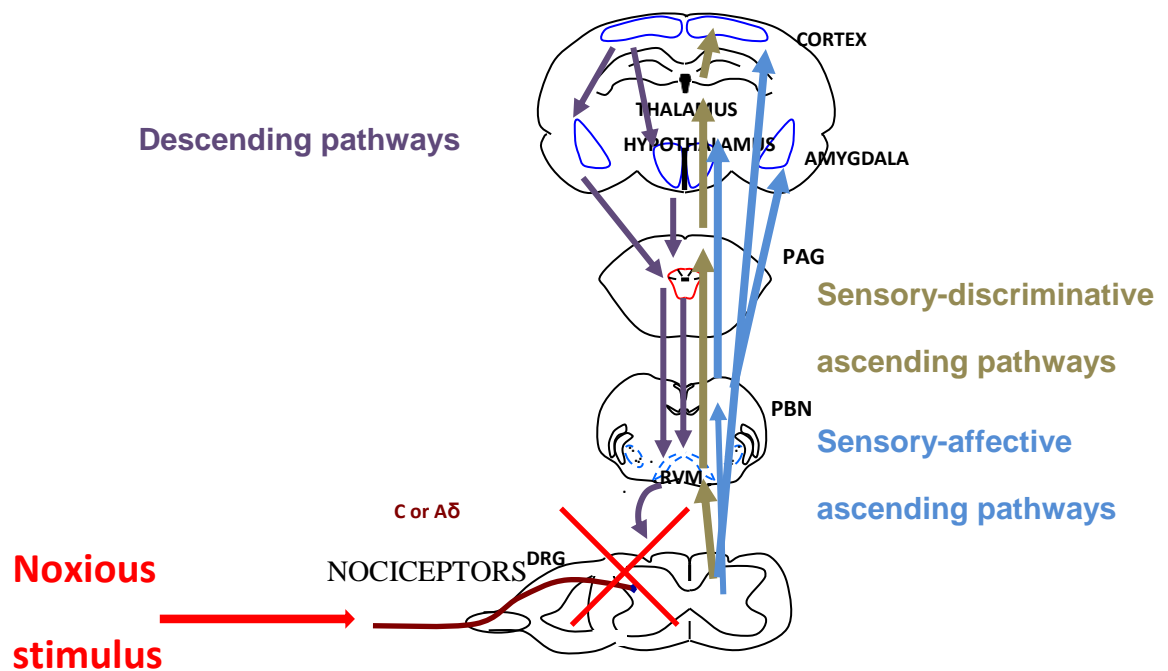


Figure 1.1: Ascending and descending pain pathways; the primary afferent neurons project and transmit the nociceptive information to the dorsal horn of the spinal cord. Nociceptive information is then relayed either directly to the cortex or indirectly to the cortex through the brainstem, midbrain, and thalamus via the ascending pain pathways. Neurons in the ventral spinothalamic pathway project onto the thalamic nuclei and then synapse with third-order neurons that are terminating in the postcentral gyrus of the cortex. The spinoparabrachial pathway terminates in the parabrachial nucleus (PBN), where they synapse with third-order neurons that project onto the hypothalamus, amygdala and cortex. The descending pain pathway originates in the cortex, hypothalamus, and amygdala, which project to the periaqueductal grey (PAG) and the rostroventromedial medulla (RVM) and finally to the dorsal horn of the spinal cord. Activation of this pathway may either inhibit or facilitate pain depending on the subset of neurons and receptors activated. The sensory-discriminative aspects of pain include quality, location and intensity processing, while affective component of pain comprises the unpleasant character of pain perception DRG: dorsal root ganglia. Figure adapted from (Olango, 2012).

1.2.1 Ascending pain pathways

Ascending pathways include spinothalamic pathways, the spinoparabrachial pathway, the spinomesencephalic, the spinothalamic and the spinoreticular pathways (Millan, 1999). The classic pain pathway usually consists of a three-neuron chain that transmits pain information from the periphery to higher brain regions (Cross, 1994). The first order neuron has its cell body in the dorsal root ganglion and two axons, one extending distally to the tissue it innervates while the other extending proximally to the dorsal horn of the spinal cord (Woolf, 2000). In the dorsal horn, this axon synapses with the second order neuron which in turn will cross the spinal cord through the anterior white commissure and ascends through the lateral spinothalamic tract to the thalamus. In the thalamus, the second order neuron synapses with the third order neuron, which ascends through the internal capsule and corona radiata to the postcentral gyrus of the cerebral cortex (Cross, 1994). The pathways project contralaterally to higher brain regions as the second order neurons traverse the midline. Neurons in the spinothalamic pathway are important in relaying information pertaining to the sensory-discriminative perception of noxious stimuli such as stimulus intensity, location, duration, temporal pattern and quality. They originate from the laminae I, II and IV-VIII of dorsal horn of spinal cord and innervate the thalamic nuclei where they synapse with third-order neurons terminating in the postcentral gyrus of the cortex (Hunt and Rossi, 1985; Millan, 1999; Willis and Westlund, 1997). The spinothalamic tract is further classified into three sub-pathways: the ventral spinothalamic tract (directly projects to nuclei of the lateral complex of the thalamus and onto the somatosensory cortex and is thus involved in the sensory–discriminative component of pain), the dorsal spinothalamic tract, (projects to nuclei of the posterior medial and intralaminar complex of the thalamus, involved in the motivational–affective aspects of pain) and the monosynaptic spinothalamic pathway (projects directly to the medial central nucleus of the thalamus involved in the affective component of the pain experience) (Almeida et al., 2004; Hodge and Apkarian, 1990; Lovick, 1996; Lumb and Lovick, 1993). The thalamus is crucial in relaying nociceptive information via the ascending pain pathways and further projects onto the cortical regions which are involved in pain perception and emotional pain processing. The spinoparabrachial pathway is involved in the cognitive-affective dimension of pain as these neurons from the dorsal horn of the spinal cord project onto the parabrachial area, where they form synaptic connections with third order neurons that terminate in the ventral medial nucleus of the hypothalamus and the central nucleus of the amygdala

(Bester et al., 1997; Derbyshire et al., 1997; Krout et al., 1998). The spinothalamic tract plays an important role in sympathetic and motivational responses to pain, as it projects onto the parabrachial nucleus and the PAG via the spinomesencephalic tract (Allen and Pronych, 1997; Jatsu Azkue et al., 1997; Keay et al., 1997). The PAG functions as a behavioural state-dependent relay station for modulation of both the descending and ascending pain pathways (Krout et al., 1998), and it also projects to the parabrachial nucleus which is known to be involved in emotional response to pain (Hunt and Mantyh, 2001; Lovick, 1996; Lumb and Lovick, 1993).

1.2.2 Descending pain pathway (control, inhibition and facilitation)

The gate control theory proposed by Melzack and Wall in 1965 states that non-painful stimulus gates the painful input at the level of the dorsal horn of the spinal cord, which prevents pain sensation from traveling to the central nervous system (Melzack and Wall, 1965). Ascending pain pathways relaying nociceptive information can also be controlled i.e. either suppressed or enhanced, by the activity of neurons within top-down descending pain pathways. These pathways originate from the hypothalamus, limbic areas or cortex and project to the PAG and then brainstem areas (e.g. the RVM and locus coeruleus) to terminate in the dorsal horn of the spinal cord (Millan, 2002) (Figure 1.1).

Descending control of pain may be either inhibitory or facilitatory. The descending inhibition of ascending pain pathways occurs at the level of the dorsal horn of the spinal cord (Beitz, 1982; Hopkins and Holstege, 1978). Neurons from the PAG project to the RVM which in turn projects to the dorsal horn of the spinal cord to modulate pain transmission via the activity of ON and OFF cells in the RVM. The amygdala, which is known to be involved in mediating defensive responses is interconnected with the PAG contributing to the descending inhibitory pain pathway (Behbehani, 1995; Hopkins and Holstege, 1978; Oka et al., 2008). Similarly the hypothalamus, limbic forebrain, and are known to modulate different behavioural responses based on the column of PAG stimulated (Keay and Bandler, 2001).

Spinal nociception is reported to be enhanced via descending facilitation which can contribute to the development and maintenance of chronic pain (Burgess et al., 2002; Kovelowski et al., 2000; Porreca et al., 2002). During acute nociception, both

descending facilitatory and inhibitory pathways are activated and are counterbalanced. However, with sustained nociceptive input, neuroplastic changes (ability of the brain to reorganize itself by forming new neural connections to compensate injury and disease conditions) in the supraspinal regions lead to facilitatory mechanisms that drive the amplified pain response. Anatomically, both the facilitatory and inhibitory pathways and sites are inseparable, with most of the supraspinal structures involved in mediating both the pathways directly or indirectly such as RVM or PAG, respectively. The RVM is directly involved in both the pathways via the RVM ON and OFF cell activity (Vanegas et al., 1984). The PAG influence spinal nociception via the RVM. Hyperalgesia or allodynia which characterizes chronic, inflammatory, neuropathic or visceral pain conditions may be mediated, at least in part, via activity in the descending facilitatory pain pathway (Asante and Dickenson, 2010; Kim et al., 2014; Pertovaara, 1998). Descending serotonergic drive is key in for pain facilitation (Kim et al., 2014; Rahman et al., 2009; Sikandar et al., 2012; Rahman et al., 2006). Serotonin (5-HT) is known to mediate nociception by increasing the release of substance P, calcitonin gene-related peptide, and neurokinin A from the central terminals of primary afferent fibres that express 5-HT₃ receptors (Inoue et al., 1997; Saria et al., 1990). It has been well documented that neuronal activity from the RVM regulates serotonin release at the spinal neurons (Millan, 2002). Descending inhibition of pain sensation includes projections from various brainstem nuclei to the spinal cord dorsal horn via the dorsolateral funiculus (DLF). The DLF fibres are serotonergic projections from the raphe nuclei. These descending fibers suppress pain transmission at the nociceptive spinal cord neurons probably by hyperpolarizing afferent sensory neurons using serotonin or other monoamines (norepinephrine) as principal inhibitory mediators (Dafny, 2008). The inhibitory action of serotonin on structures of the dorsal horn may also be influenced by activation of opioid-releasing interneurons. In preclinical models, naloxone, an opioid antagonist, decreased the analgesic effect of intraspinal serotonin and *vice-versa* with serotonin antagonists blocking the analgesic effects of morphine near the spinal cord (McHugh and McHugh, 2000). In rats, antidepressants and antagonists of monoamine receptors treatment decreased formalin-evoked nociceptive behaviour, indicating functional interactions between noradrenergic and serotonergic neurons as mechanisms of antidepressant-induced pain-control (Yokogawa et al., 2002).

The RVM is known as a heterogeneous region incorporating several nuclei which provide descending pathways to both superficial and deep dorsal horn laminae. It is

subdivided into medially, the nucleus raphe magnus and more dorsally and/or laterally, the nucleus reticularis gigantocellularis, the nucleus gigantocellularis pars alpha and the nucleus reticularis paragigantocellularis lateralis (Azami et al., 2001; Basbaum and Fields, 1984; Fields et al., 1991; McNally, 1999; Urban and Gebhart, 1999; Urban et al., 1999a, 1999b; Urban et al., 1999; Watkins et al., 1998; Wei et al., 1999). OFF cells are excited by opioids indirectly and inhibited by nociceptive input: they display a transient interruption in their discharge immediately prior to a nociceptive reflex and are known to participate in descending inhibition. ON cells are inhibited by opioids and excited by nociceptive input and are known to facilitate the descending pain pathway (Bederson et al., 1990; Fields et al., 1991; Gao and Mason, 2000; Kaplan and Fields, 1991; Mason, 1999; Zhuo and Gebhart, 1997, 1992). Thus, neuroplastic changes in supraspinal regions such as the RVM and PAG might underlie the hyperexcitability of spinal dorsal horn neurons wherein altered excitatory drive is mediated by 5-HT acting on spinal 5-HT₃ receptors.

1.3 Hyperalgesia associated with negative affective state

There is a high prevalence of comorbidity of psychiatric disorders with chronic pain states, and brain regions and neural substrates mediating both overlap considerably (Burke et al., 2015; Fitzgibbon et al., 2016; Jennings et al., 2014). A clinical study in 2012 reported that 77% of subjects with chronic pain along with generalised anxiety disorder reported the onset of anxiety disorder prior to chronic pain suggesting that the affective component may predispose to the development of chronic pain (Knaster et al., 2012). Furthermore, a study by Keogh and Cochrane reported that pain threshold was reduced in patients with anxiety disorders (Keogh and Cochrane, 2002). Clinical studies reported that there is a high prevalence of depression in patients suffering from chronic pain (Bair et al., 2003; Raftery et al., 2011). In addition, the current use of drugs such as pregabalin, amitriptyline and duloxetine for the treatment of both pain and anxiety/depression further explains the close associations that exist between these disorders. Increased understanding of common neural substrates and mechanisms is important.

1.3.1 Models of hyperalgesia associated with negative affective state

There is evidence that exposure to chronic or repeated stress can produce maladaptive neurobiological changes in pathways associated with pain processing, resulting in stress-induced hyperalgesia (SIH). Preclinical animal models have been employed for the study of SIH, these models mainly involve the repeated or persistent application of a for several days to weeks, in combination with a test for pain responding. These rodent models offer a greater scope to study the impact of different types of stressors on pain responding and the underlying neural substrates and neurobiological mechanisms involved. Preclinical studies of SIH are essential for our understanding of the mechanisms underpinning stress-related pain syndromes and for the identification of neural pathways and substrates, and the development of novel therapeutic agents for their clinical management. The models of SIH have been described in detail in the Table 1.1 below and also further information is available in (Jennings et al., 2014).

Species, sex and Strain	Frequency/ Duration	Pain Test	Pain behaviour observations	Reference
Forced Swim Stress				
		Formalin test and hot plate test	Inflammatory and thermal hyperalgesia	(Quintero et al., 2011, 2003, 2000; Suarez-Roca et al., 2008, 2006b)
Male Sprague-Dawley rats	Day 1: 10mins Day 2-3: 20mins	Carrageenan intra-muscular injection followed by grip strength	Mechanical hyperalgesia as determined by reduced grip strength	(Suarez-Roca et al., 2006b)
Male Swiss albino mice	Two swim stress sessions 6mins duration, 8hrs apart	Hot plate	Thermal hyperalgesia	(Suaudeau and Costentin, 2000)
Male Wistar rats	5 minute sessions daily for 5 days	Tail flick test	Thermal hyperalgesia	(Fereidoni et al., 2007)
Adult female Swiss Webster mice	15 daily swims	Tail flick test and grip strength	Thermal and mechanical hyperalgesia	(Abdelhamid et al., 2013)
Male albino mice	6 minute sessions daily for 15 days	Tail immersion test	Thermal hyperalgesia	(Dhir and Kulkarni, 2008)

Repeated Cold Stress/ Specific alternation rhythm of temperature (SART)

Male 4-week old ddY mice	Over 7 days: Alternating 24°C/ 4°C every hour for 7 hours; 4°C for final 17 hours	Randall-Selitto apparatus	Mechanical hyperalgesia (days 5-7)	(Ohara et al., 1991)
Male Sprague-Dawley rats	Over 5 days: Alternating 24°C/ 4°C or -3° every 30 mins for 7 ½ hours; 4°C/-3° for final 16 ½ hours	Randall–Selitto test and the von Frey hair test	Mechanical hyperalgesia (greater in -3°C group than 4°C group)	(Nasu et al., 2010)
Male Wistar Rats	Over 5 days: Alternating 24°C/-3° every hour for 4 hours; -3° for remaining 20 hours	Randall–Selitto test	Mechanical hyperalgesia	(Fujisawa et al., 2008)
Male Wistar rats	Over 5 days: Alternating 24°C/ -3° every 2 hours for 6 hours; -3° for remaining 18 hours	Footshock on one of two floors	Decreased escape latency	(Kawanishi et al., 1997)

Restraint Stress

Male and female (mixed estrous phases) Wistar rats	Daily 1 hr restraint for 40 days	Tail flick test	Thermal hyperalgesia in males, no effect in females	(Gamero et al., 1998)
	<u>Acute</u> : 15mins, 30mins or 1hr	Formalin injection into the	Increased inflammatory	(Gameiro et al., 2006)

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Male Wistar rats	restraint <u>Subchronic</u> : 1hr restraint for 3 days <u>Chronic</u> : 1h daily, 5 days per week for 40 days	temporomandibular joint (TMJ)	pain in chronic restraint stress rats	
Male Sprague-Dawley rats	Daily 1hr restraint for 4 days over for 5 weeks	von Frey, Randall-Selitto, Tail immersion test, Acetone-induced cold allodynia, Formalin test	Inflammatory, thermal and mechanical hyperalgesia	(Bardin et al., 2009)
Adult male Wistar rats	1hr daily restraints for 5 days per week over 8 weeks	Tail flick test ^a Formalin injection into TMJ ^b	Thermal and inflammatory hyperalgesia	(da Silva Torres et al., 2003; Gameiro et al., 2005)
Male Sprague-Dawley rats	6hr restraint once or over 1, 2 or 3 weeks	Tail flick test	Thermal hyperalgesia following 2 and 3 week restraint	(Imbe et al., 2004)
Male Sprague ^a Dawley Rats Male and female Wistar rats ^b	Acute restraint for 2hrs in restraint cage	Colorectal distension	Visceral hyperalgesia	(Eutamene et al., 2010; Ohashi-Doi et al., 2010)
Male Sprague-Dawley rats	2hr restraint stress 4 days	Colorectal distension	Visceral hyperalgesia	(Shen et al., 2010)
Male Wistar rats	1hr restraint 5 days a week for 11 weeks	von Frey Test and hot plate	Mechanical allodynia and thermal hyperalgesia	(Spezia Adachi et al., 2012)

Immobilisation Stress

Adult male Sprague-Dawley rats	90 mins daily for 7 days	Tail-Flick test	Thermal hyperalgesia	(Costa et al., 2005)
Male ICR mice	1hr daily for 5 days	Formalin test	Inflammatory hyperalgesia	(Seo et al., 2006)

Social Defeat

Male Sprague-Dawley Rats (Long Evans rats as intruder)	Four daily intruder sessions divided into two periods (see above)	von Frey, Randall–Selitto test and formalin test	Mechanical and inflammatory hyperalgesia	(Rivat et al., 2010)
Male Long-Evans Rats	Resident rats were vasectomised prior to testing. Five daily intruder sessions divided into two periods (see above)	Formalin test ^a , thermal preference and thermal escape tests ^b	Inflammatory and thermal hyperalgesia	(Andre et al., 2005; Marcinkiewicz et al., 2009)

Water Avoidance

Male Wistar Rats	1hr per day for 10 consecutive days	Colorectal distension	Visceral hyperalgesia	(Bradesi et al., 2009, 2007, 2006, 2005; He et al., 2013; Larauche et al., 2008)
Male Sprague-Dawley rats	1hr per day for 10 consecutive days	von Frey test ^a , colorectal distension ^b	Mechanical and visceral hyperalgesia	(Chen et al., 2011; Green et al., 2011b)

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Adult male C57Bl/6 mice	1hr per day for 10 consecutive days	Colorectal distension	Visceral hyperalgesia	(Hong et al., 2009a; Larauche et al., 2010)
Male Sprague-Dawley Rats	Over Three days: Tones played over four frequencies over 30 minute time period	Randall Selitto test ^a Colorectal distension ^b	Mechanical and Visceral hyperalgesia	(Green et al., 2011b; Khasar et al., 2009, 2005)

Chronic Mild Stress

Male Wistar Rats	Unpredictable Chronic stress for 6 weeks;	von Frey and hot plate in normal and complete Freund's adjuvant chronic pain rat model and formalin test	Increased mechanical and thermal thresholds and inflammatory hyperalgesia	(Shi et al., 2010a)
Male Wistar Rats	Unpredictable Chronic stress for 6 weeks;	Hot Plate and von Frey tests in naive and SNL rats	Increased thermal and inflammatory pain thresholds for both normal and SNL rats	(Shi et al., 2010b)

Maternal Separation /Deprivation/ Early life stress

Wistar male rats ^a Sprague-Dawley ^b male rats	Pups separated from mother for 180 minutes from days 2-14	Colorectal distension	Visceral hyperalgesia	(E.K.Y. Chung et al., 2007; K.Y. Chung et al., 2007; Zhang et al., 2008, 2009)
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Long-Evans rats	Pups separated from mother for 180 minutes from days 2-14	Colorectal distension	Visceral hyperalgesia	(van den Wijngaard et al., 2012; Welting et al., 2005; Wouters et al., 2012)
Wistar male and female rats	24 hours MD on PND 9	Hot Plate, von Frey, acetone test and prior to and after spinal nerve ligation (SNL)	Thermal hypoalgesia, mechanical allodynia in females	(Burke et al., 2013)
Sprague-Dawley rats	Mother and pups are placed in cages fitted with a stainless steel mesh bottom on post-natal day 2 to 9.	Digital force transducer	Mechanical hyperalgesia	(Alvarez et al., 2013; Green et al., 2011a)

Noise Stress

Male Sprague-Dawley rats	105 dB tone of mixed frequencies, ranging from 11 to 19 kHz over 30 minutes over 3 to 4 days	Paw-withdrawal threshold	Enhanced inflammatory pain	(Khasar et al., 2009, 2005)
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Vibration Stress

Male and female Wistar rats	4 Hz applied to restraint tube for 5 min	Tail flick test	Hyperalgesia developed at 2-10 minutes after stress in male rats. Thermal hyperalgesia and female responding was oestrus dependent	(Devall et al., 2011, 2009)
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PTSD model				
Male Sprague–Dawley rats	2 hr restraint, 20 mins swim followed by 15 min rest, inhalation of an ether until unconscious	von Frey test and paw withdrawal to heat stimulus	Mechanical allodynia and thermal hyperalgesia	(Zhang et al., 2012)
Male Sprague–Dawley rats	2 hr restraint, 20 mins swim followed by 15 min rest, inhalation of an ether until unconscious then footshock when conscious	Colorectal distension	Visceral hyperalgesia	(He et al., 2013)
WKY rat				
Male Wistar-Kyoto Rats	Inbred rat strain	Colorectal distension, intra-plantar formalin injection, CFA injected into the temporomandibular joint, CCI	Visceral hyperalgesia, enhanced inflammatory pain responding, exacerbated mechanical allodynia	(Burke et al., 2010; Greenwood-Van Meerveld et al., 2005; Gunter et al., 2000; O’ Mahony et al., 2013; Rea et al., 2014; Zeng et al., 2008)

Table 1.1: Rodent models of hyperalgesia associated with negative affect/stress. Table adapted from (Jennings et al., 2014). CFA: Complete Freund’s adjuvant, CCI: Chronic constriction injury, MD: Maternal deprivation, PND: Post-natal day, SNL: Spinal nerve ligation, TMJ: Temporomandibular joint

Critical scrutiny of these data suggests that the nature, duration and intensity of the stressor are key determinants of the effects of stress on pain. Stress and anxiety, depending on their nature, duration and intensity, can exert potent, but complex, modulatory influence either by reducing or exacerbating the pain. The animal models that have been developed have facilitated an increased understanding of the neurobiology of SIH. It is also worth considering which of these animal models most appropriately depicts SIH clinically. At this juncture, it is quite difficult to single out any individual animal model as being closest to the expression of SIH in humans because the mechanism underlying the latter are still poorly understood. However, social defeat, chronic mild stress and early life stress models may have greater translational value. However, when coupled with a chronic pain model, their reproducibility can be more challenging than models that use a more robust, acute/sub-acute stressor. It is well known that different models engage different neural substrates and the various neural substrates and neurotransmitter systems involved in SIH are discussed later on in this chapter. In these studies, mostly male rats have been used and the question of sexual dimorphism with respect to SIH still needs to be addressed. Employing techniques such as positron emission topography in these aforementioned preclinical studies could further enhance our level of understanding the of neuroanatomical substrates underlying stress-pain interactions.

In this thesis, WKY rats in the formalin test are used to study the effect of negative affective state on pain. The formalin test is a model of tonic, persistent pain resulting from formalin-induced tissue activation of primary afferent neurons and inflammation. It is a useful model, particularly for the screening of novel compounds, since it encompasses inflammatory, neurogenic and central mechanisms of nociception (Tjølsen et al., 1992). The formalin model is used to study the mechanisms of persistent inflammatory pain. Additional animal models of inflammatory hyperalgesia that mimic human clinical pain conditions have been developed by the injection of inflammatory agents into the rat hind paw (Hargreaves et al., 1988; Iadarola et al., 1988). Unlike traditional reflex tests of nociception (e.g. tail-flick, hot-plate), pain produced by the hind paw injection of formalin results from both the direct chemical-induced activation of primary afferent fibres and from inflammatory processes associated with tissue damage and, thus, more closely resembles clinical pain conditions (Abbott et al., 1999; Dubuisson and Dennis, 1977; Murray et al., 1988). The intraplantar injection of a formalin solution produces nociceptive behaviors, which appear in two distinct phases.

The first (acute) phase begins at the time of injection and lasts for about 5-10 min. The subsequent, second (tonic) phase starts at approximately 10 min postinjection. The first phase is thought to result from a direct chemical activation of nociceptive afferent fibers. The second phase is believed to be mediated by the activation of central sensitized neurons due to peripheral inflammation as well as ongoing activity of primary afferents (Dubner and Ren, 1999; Hunzkaar and Hole, 1987; Puig and Sorkin, 1996). Rea et al., from our laboratory, demonstrated that WKY rats have reduced formalin-evoked AEA and 2-AG levels in the RVM compared to SD counterparts. In the same study, intra-RVM administration of the FAAH inhibitor URB597 attenuated enhanced formalin-evoked nociceptive behaviour in WKY rats, suggesting impaired endocannabinoid mobilisation but not CB₁ receptor insensitivity in the RVM of hyperalgesic WKY rats (Rea et al., 2014). Jennings et al provides evidence that the WKY model of negative affect is insensitive to a CB₁ receptor agonist administered directly into all three columns of the PAG (Jennings, 2015). Previous studies suggest that TRPV1 and CB₁ receptors exert opposite effects on anxiety-related behaviour (Casarotto et al., 2012a; Uliana et al., 2016) conditions. Thus, taken together the previous work on CB₁ in the PAG-RVM pathway suggests that altered expression and/or functionality of TRPV1 may underly the hyperalgesic behaviour in WKY rats, as TRPV1 and CB₁ are known to maintain homeostasis.

1.3.2 WKY rat model – Genetic model of hyperalgesia associated with negative affective state

Genetics is an important factor influencing hyperalgesia associated with negative affective state. The Wistar-Kyoto (WKY) rat is an inbred strain developed from Wistar rats (Okamoto and Aoki, 1963) which was developed as a control strain for studies with a rat model of hypertension (spontaneously hypertensive [SHR] rats;), but was found to exhibit hyperresponsivity to stressful stimuli when compared to other rat strains such as Wistar and Sprague Dawley (SD) strains. One of the main advantages of using an inbred strain to study stress-related changes in physiology is that it enables a researcher to tease out the environmental and genetic components of the trait. Examining behavioural changes in the WKY rat has revealed that this strain of rat displays depressive-like behaviour (increase in immobility in forced-swim test [FST]) and high anxiety-like behaviour (decrease in time spent in the open arms of elevated plus maze [EPM] and inner circle of open field [OF]) when compared to SD and Wistar

counterparts (Gentsch et al., 1987; Malkesman and Weller, 2009; Tejani-Butt et al., 1994). Hypothalamic-pituitary-adrenal axis (HPA) and hypothalamic-pituitary-thyroid axis (HPT) are reported also to be dysregulated in WKY rats (De La Garza and Mahoney, 2004; Ma and Morilak, 2004). Thus, WKY rats have significantly higher basal plasma adrenocorticotrophic hormone and corticosterone levels for several hours after diurnal peak when compared to the Wistar rats. Furthermore, higher corticosterone levels are reported in WKY rats in response to acute stress when compared to SD rats (Solberg et al., 2001). In the PFC and nucleus accumbens, which are important in regulating anxiety-like behaviour and depressive-like behaviour, 5-HT and dopamine release are impaired in WKY rats at baseline levels (De La Garza and Mahoney, 2004).

Studies from our laboratory have demonstrated that WKY rats exhibit thermal hyperalgesia when compared to SD rats in the hot plate test (Burke et al., 2010; Olango, 2012). In contrast, WKY rats respond similarly to SD rats in the tail flick test (Burke et al., 2010; Taylor et al., 2001), thus the response to thermal noxious stimuli may depend on the test conditions under investigation. Several studies have demonstrated that WKY rats exhibit hyperalgesia to colorectal-distention (Meerveld et al., 2005; Gunter et al., 2000; O' Mahony et al., 2013) and thus are now regarded as an animal model of irritable bowel syndrome (IBS). Furthermore, data from our lab have demonstrated that WKY rats display enhanced inflammatory pain responding compared to SD rats following intra-plantar formalin injection (Burke et al., 2010, Rea et al., 2014). Accordingly, hyperalgesia was also observed in WKY rats following complete Freund's adjuvant (CFA) injection into the temporomandibular joint (Wang et al., 2012). In the chronic constriction injury model of neuropathic pain, WKY rats exhibited enhanced mechanical allodynia when compared to the Wistar rats (Zeng et al., 2008). Studies have also reported that hyperalgesia is exacerbated upon application of a water avoidance stress paradigm in WKY rats (Robbins et al., 2007).

Thus the WKY rat exhibits both a negative affective and hyperalgesic phenotype, and so is a useful model for investigating the influence of genetic background on hyperalgesia associated with negative affective state. It should also be noted that the depressive-like behaviour in WKY rats is not altered by traditional antidepressant (selective serotonin reuptake inhibitors (SSRI) and tricyclic antidepressants (TCA)) administration (Abdeljalil Lahmame et al., 1997; López-Rubalcava and Lucki, 2000; Tejani-Butt et al., 2003) suggesting that they may represent a model of treatment-resistant major

depressive disorder. The lack of effect of these antidepressants cannot be explained by pharmacokinetic differences as serum imipramine levels after 15-day treatment were higher in WKY than in SD and Brown–Norway rats (Lahmame et al., 1997) although this treatment only attenuated immobility in the FST in SD and Brown-Norway but not WKY rats. This suggests a sub-sensitivity to imipramine (and possibly other antidepressant) treatment observed in WKY rats.

Gene expression studies with the help of microarray technology identified the differential gene expression between WKY rats and SD rats in the locus coeruleus (LC) and dorsal raphe (DR) which are key sites of origin for noradrenergic and serotonergic pathways, respectively (Pearson et al., 2006). Differences in gene expression in LC or DR neurons could underlie differential responses of these systems to stress and account for the stress-sensitive phenotype of the WKY strain. The stress-sensitive phenotype might be attributed to higher expression of genes encoding for noradrenaline synthesis or metabolism that were reported in WKY rats. Along with the genetic impairment of noradrenergic systems, an increased expression of genes encoding opioids, and orexin (hypocretin) which regulates the neurotransmitters that convey afferent information to the LC was reported. The low expression of genes encoding cytoskeletal proteins that are important for structural plasticity and several potassium channels, suggests that the excitability and synaptic integration of DR neurons may be altered in WKY rats (Pearson et al., 2006).

Studies suggested a role for kappa opioid receptors in the hyperresponsivity to stress and not pain behaviour of WKY rats. The kappa opioid receptor antagonist DIPPA exhibited antidepressant-like behaviour in FST (Carr et al., 2010) and anxiolytic behaviour in novelty-induced hypophagia in WKY and SD rats (Carr and Lucki, 2010). In tail withdrawal test, however μ -opioid partial agonist and κ -opioid antagonist, buprenorphine had no effect in WKY rats (Avsaroglu et al., 2007).

Recently the role of the endocannabinoid system has been studied in WKY rats, wherein the endocannabinoid AEA (Anandamide), fatty acid amide hydrolase (FAAH—the enzyme responsible for the catabolism of AEA), cannabinoid₁ (CB₁) receptor and brain-derived neurotrophic factor (BDNF) levels were analysed using immunoblotting in key regions such as prefrontal cortex (PFC) and hippocampus (Vinod et al., 2012). BDNF which is known to modulate neuroplasticity binds to TrkB receptors, which are transactivated by endocannabinoids (Berghuis et al., 2005). Further, cannabinoids alter

BDNF expression via actions on the extracellular signal-regulated kinase (ERK) signaling pathway (Derkinderen et al., 2003; Rubino et al., 2006). WKY rats had higher levels of FAAH and CB₁ in the PFC and hippocampus and lower levels of AEA and BDNF when compared to the Wistar strain. The levels of BDNF were lower in PFC and hippocampus, and pharmacological blockade of FAAH elevated the BDNF levels, likely contributing to the antidepressant activity of FAAH inhibition in WKY rats in the FST and sucrose preference tests (Vinod et al., 2012). Therefore, endocannabinoid tone might be a viable target for treatment-resistant depression. However, it remains to be determined if this system may also modulate hyperalgesia observed in the model and thus provide a means of treating hyperalgesia associated with negative affect.

1.3.3 Role of CNS in hyperalgesia associated with negative affective state

The CNS plays a crucial role in nociceptive transmission as discussed earlier. This nociceptive transmission is influenced by stress and negative affect via altered neuronal activity in key substrates including the cortex, amygdala, PAG, RVM and spinal cord. In this section, these key components of the CNS and their role in hyperalgesia associated with negative affective state will be considered.

1.3.3.1 Cortex

The cortex plays a pivotal role in both the ascending and descending pain pathways. Neuroimaging studies report that chronic pain and psychiatric conditions are associated with structural and functional reorganisation of cortical structures (Asami et al., 2008; Burgmer et al., 2009; Flor et al., 2001; Hayes et al., 2012; Karl et al., 2001; Pleger et al., 2006), especially the primary somatosensory cortex (S1) in chronic pain conditions using functional magnetic resonance imaging (Vartiainen et al., 2009; Wrigley et al., 2009). Preclinical animal studies have also demonstrated that MS (maternal separation) rats exposed to WAS (water-avoidance stress) displayed an increased in activation of the S1, suggesting that stress in the adult MS rat results in enhanced sensory input to visceral noxious stimulation (Mendes-Gomes et al., 2011).

Neuroplastic changes in the grey matter have been reported in patients suffering from chronic pain (Desouza et al., 2013). Sub-cortical structures such as the anterior cingulate cortex and the insular cortex are important in modulating the sensory and affective aspects of pain (Basbaum et al., 2009; Xie et al., 2009). Clinical study reported

that individuals with lower pain thresholds exhibit recurrent pain-induced activation of the S1 cortex, ACC and PFC compared to individuals with higher thresholds (Coghill et al., 2003). Healthy volunteers experienced higher pain perception when stimulated with electrical impulses in a negative context, an effect associated with activation of the ACC (Senkowski et al., 2011; Yoshino et al., 2012). Cortical structures such as the insula, mid-cingulate and ventrolateral PFC are activated in IBS patients upon exposure to a psychological stressor (Elsenbruch et al., 2010). Preclinical studies reported similar activity in the insular cortex, where exposure to a psychological stressor (Water avoidance stress (WAS) model) was associated with colorectal distension (Wang et al., 2013). In the same study, reduced activation of the prelimbic area of the PFC was reported.

Furthermore, substantial evidence from preclinical studies demonstrates that electrical stimulation of the ACC increases C-fibre activation in response to noxious thermal stimulation in the Hargreaves test (Zhang et al., 2005) and lesioning of the same region prevented formalin-induced conditioned place avoidance (Gao et al., 2004, Johansen et al., 2001), implicating the ACC in pain associated with negative affective state. SNI (spared nerve injury) model of neuropathic pain was associated with a decrease in the volume of ACC and insula, and in anxiety-related tests (EPM and OF) indicated a decrease in volume of PFC correlated with the behavioural changes (Seminowicz et al., 2009).

1.3.3.2 Amygdala:

Fear and anxiety which are processed by the amygdala can modulate nociceptive processing. (Butler and Finn, 2009; Rhudy and Meagher, 2000). Clinical studies have shown that via a positron emission tomography (PET) study, that reduced μ -opioid receptor expression in the amygdala of individuals in continuous pain (Zubieta et al., 2001). Preclinical work has demonstrated that anxiety-like behaviour in mouse models of SNL (spinal nerve ligation) and CFA (Complete Freund's Adjuvant) an effect associated with reduced binding (decrease in opioidergic function) of μ - and δ - opioid receptor in the amygdala (Narita et al., 2006). In the same study, the κ -opioid receptor was unaffected in the SNL but reduced in the CFA model (Narita et al., 2006). Implantation of micropellets loaded with corticosterone into the central amygdala (CeA) resulted in visceral hyperalgesia in the WAS model, and no such effect was seen when the micropellets were loaded with glucocorticoid receptor antagonist mifepristone, or

the mineralocorticoid receptor antagonist spironolactone (Myers et al., 2007; Myers and Greenwood-Van Meerveld, 2012, 2010, 2007). Higher expression of corticotropin-releasing factor receptor 1 (CRF1) receptors in the basolateral amygdala (BLA) were reported in WKY rats when compared to SD rats, (Bravo et al., 2011). Furthermore, to further investigate the role of the amygdala in relation to hyperalgesia associated with negative affective state, WKY rats were administered with CRF1 antagonist (CP - 376395) micropellets in CeA and this treatment blocked the colonic hypersensitivity (Johnson et al., 2012). Intra-CeA administration of mifepristone or spironolactone had no effect on the colonic hypersensitivity in the WKY rats (Johnson et al., 2012).

1.3.3.3 Periaqueductal grey (PAG):

The PAG is the major focus of the work described in this thesis. The PAG refers to the region of the midbrain that surrounds the cerebral aqueduct. The most rostral region of the PAG is at the level of the posterior commissure and the most rostral level of the third nucleus. The most caudal PAG level is at the level of the dorsal tegmental nucleus.

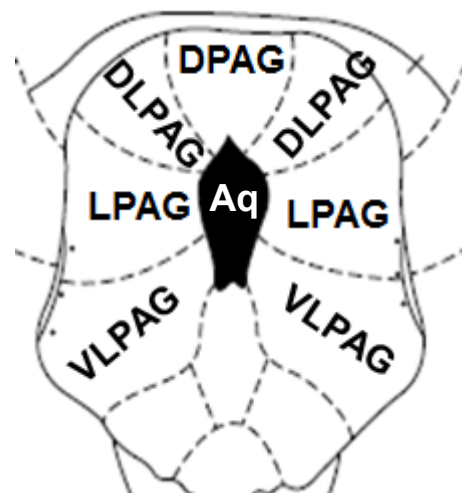


Figure 1.2: Divisions of PAG. DPAG dorsomedial PAG; DLPAG-dorsolateral PAG; LPAG-lateral PAG; VLPAG-ventrolateral PAG; Aqueduct; Figure adapted from (Paxinos and Watson, 1998).

Originally, the human PAG was divided into three regions, the dorsal, medial and ventral subregions (Olszewski and Baxter, 1954). Liu and Hamilton subdivided the PAG of the cat into dorsal, medial and lateral subdivisions (Liu and Hamilton, 1980). On a similar basis, the morphological subdivisions within the rat PAG by using a

statistical cluster analysis system was carried out by Beitz and colleagues in rats (Beitz, 1985) (refer to Figure 1.2). In addition, both qualitative and quantitative data concerning neuronal size, shape, and density were obtained. The data obtained from cluster maps suggested the presence of four subdivisions within this midbrain region of rat i.e. dorsal, dorsolateral (DLPAG), medial (LPAG) and ventrolateral (VLPAG) divisions (Beitz, 1985). Neuronal density decreased from rostral to caudal in the PAG. The medial subdivision contains the smallest neurons and exhibits the lowest cell density whereas the dorsolateral and ventrolateral divisions contain the largest neurons while the dorsal division displays the highest density in midbrain region of rats (Beitz, 1985). The somata of neurons in the PAG range between 10 and 35µm with occasional cells with somata of 35-40µm (Beitz, 1985; Mantyh, 1982). Five types of cells have been detected in the PAG: the fusiform neurons that have one or several dendritic processes, multipolar neurons that have a very large number of dendritic arborisations, the stellate cells that have 3-6 processes, pyramidal cells with very diffuse dendritic arborisation and ependymal cells that line the cerebral aqueduct (Beitz, 1985; Liu and Hamilton, 1980; Mantyh, 1982).

The PAG is a key substrate in top-down modulation of nociception and also plays a key role in defensive/ anxiety behaviour, although not all regions of the PAG are involved in both responses (see section 1.5 entitled TRPV1 and its role in negative affective state). For example, lesioning of ventrolateral PAG (VLPAG) decreased nociceptive behaviour, but had no effect on emotional behaviour associated with pain (Mendes-Gomes et al., 2011). The PAG activation increased in the presence of colorectal distension before and after WAS. MS rats displayed enhanced nociceptive behaviour to colorectal distension in adulthood (Devall et al., 2011). Unfortunately, MS-induced alteration cannot be concluded because a cohort of non-MS animals was not included in the study. Furthermore, WAS did not affect PAG responses to colorectal distension. Fos mRNA and protein are used to identify neuronal activation in the brain and has led to many studies that have examined the detailed molecular and cellular mechanisms of Fos promoter activation (Cohen and Greenberg, 2008; Herdegen and Leah, 1998; Morgan and Curran, 1991). Stress-induced hyperalgesia induced following exposure to anxiogenic vibration resulting in a decrease in tail flick latency, was associated with decreased *c-Fos* expression in the lateral, dorsolateral and ventrolateral columns of the PAG when compared to the controls (Devall et al., 2011). Thus acute stress-induced hyperalgesia was associated with reduced neuronal activation in several columns of the

PAG. In comparison, repeated exposure of vibration stressor resulted in a 4-fold increase of *Fos* expression, particularly in the ventral part of the caudal PAG, but also in the lateral and dorsolateral regions. Thus, these results suggest that stress exposure can produce differential neurological activation in the PAG in response to acute vs repeated exposure. Further studies are required to elucidate the role of the PAG in the stressful event and at what time point the changes in the neurons occur resulting in opposite effects. In a further study, chronic restraint stress-induced hyperalgesia was associated with reduced expression of the glial fibrillary acidic protein (GFAP), an astrocyte biomarker, and the excitatory amino acid transporter 2 (EAAT-2) in the PAG (Imbe et al., 2012)). Furthermore, hyperalgesia observed in a mouse model of spared nerve injury following exposure to restraint stress, was associated with increased the mRNA expression of GFAP, BDNF and interleukin (IL)-1 in the PAG (Norman et al., 2010). These data suggest that the directionality of GFAP expression may depend on the pain model used. Together, these studies indicate a role for the PAG in stress-induced hyperalgesia.

1.3.3.4 Rostral ventromedial medulla

The RVM contains three types of cells; the ON cells, OFF cells and Neutral cells. The ON cells facilitate pain and OFF cells inhibit the pain, while the function of the neutral cells is poorly understood (Vanegas et al., 1984). Neurons project from the RVM to the spinal cord and trigeminal nucleus and their activation can result in either analgesia or hyperalgesia (Aicher et al., 2012) based on the receptors activated, type of pain model and neuronal subtypes activated (Heinricher et al., 2009).

Increased phospho-ERK-immunoreactive neurons and a decrease in GFAP and upregulation of tryptophan hydroxylase in the RVM (Fig.1.3) were associated with chronic restraint-induced thermal hyperalgesia in rats (Imbe et al., 2013). The RVM is hypothesized to mediate descending facilitation of pain in the forced swim-induced hyperalgesia in the formalin test, which in turn is attenuated after ibotenic acid-induced lesion of the RVM (Imbe et al., 2010). A cholecystinin (CCK)2 receptor antagonist administered directly into the RVM also blocked the stress-induced anxiety and hyperalgesia in repeated social defeat model of SD rats (Rivat et al., 2010). But the CCK2 receptor antagonist (YM022) injected into the RVM had no effect on stress-induced thermal hyperalgesia. Selective ablation of neurons expressing μ -opioid

receptors in the RVM with dermorphin-saporin prevented the development of whisker pad stimulation stress-induced mechanical hyperalgesia (Reynolds et al., 2011)

The results suggest that μ -opioid receptor-expressing neurons in the RVM mediate the hyperalgesia associated with negative affective state (Figure 1.3). Data from our laboratory demonstrated that pharmacological blockade of CB₁ receptors increased formalin-evoked nociceptive behaviour in WKY rats and blockade of FAAH (enzyme catabolising AEA) decreased the hyperalgesia associated with intra-plantar formalin-administration (Rea et al., 2014). In a separate study, adult Wistar rats exposed to MD (maternal deprivation), exhibited higher levels of tyrosine kinase receptor B in the RVM, which is associated with an increase in visceral hypersensitivity to colorectal distension (Chung et al., 2009). The authors demonstrated no effect on the BDNF expression levels in RVM (Chung et al., 2009), although other changes remain to be investigated. The evidence suggests that the RVM does play a role in hyperalgesia associated with negative affective state.

1.3.3.5 Spinal Cord

Persistent pain is due to alterations in the spinal circuitry leading to greater and more prolonged pain perception (Basbaum, 1999). Rats exposed to the FST displayed greater formalin-evoked nociceptive behaviour along with higher *c-Fos* expression in the dorsal horn of the spinal cord (Quintero et al., 2011, 2003). These authors have also shown that glutamate levels increased and GABA levels decreased in the spinal cord of rats exposed to the FST (Quintero et al., 2003, 2011). The hyperalgesic response and spinal glutamate levels were reversed after administration with the anxiolytic drug diazepam, suggesting a role for glutamate in the spinal cord in mediating the hyperalgesia associated with negative affective state (Quintero et al., 2011). Increased formalin-evoked nociceptive behaviour in rats exposed to FSS (3 days) was associated with increased *c-Fos* expression in the dorsal horn of the spinal cord and increased levels of glutamate and decreased levels of GABA in the lumbar spinal cord. Prior administration of the anxiolytic diazepam inhibited the hyperalgesic response and prevented changes in spinal glutamate levels (Quintero et al., 2011). Cyclooxygenase-2 and nitric oxide synthase transiently increased in the spinal cord of socially defeated rats that displayed mechanical hyperalgesia. The effect was countered with the administration of aspirin

(Rivat et al., 2010). These studies suggest that stress affects the nociceptive processing in the dorsal horn either directly or indirectly via the top-down modulation of pain.

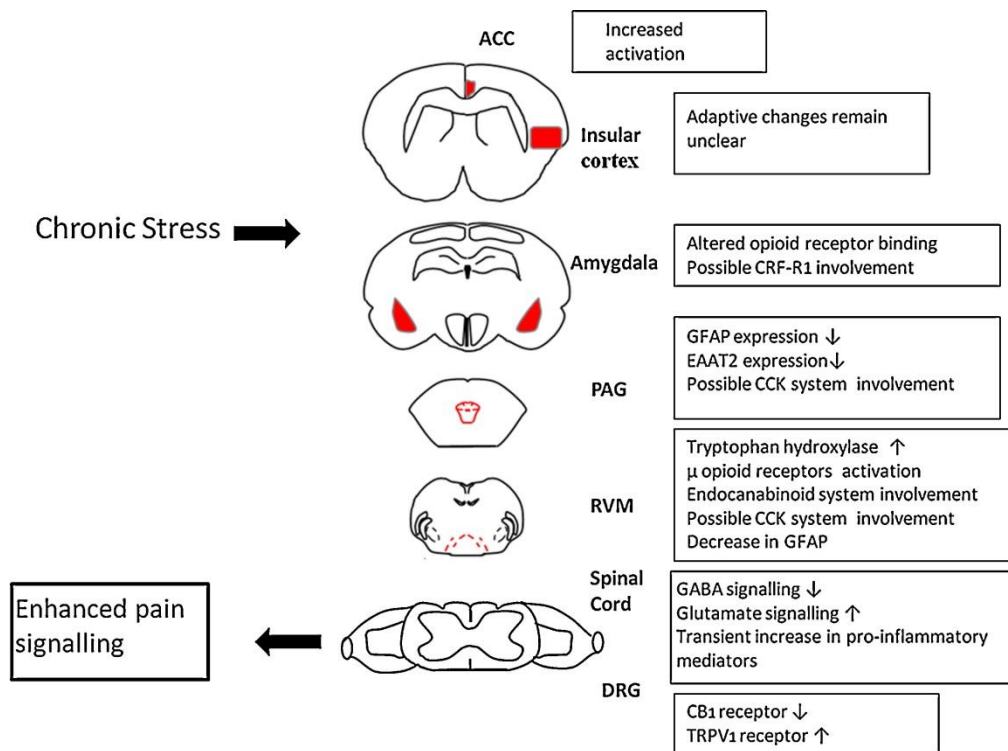


Figure 1.3: Summary of some CNS sites and neurobiological mechanisms mediating hyperalgesia associated with negative affect. ACC (anterior cingulate cortex), PAG (periaqueductal grey), RVM (rostral ventromedial medulla), DRG (dorsal root ganglia), pERK (phosphorylated extracellular signal-regulated kinase), 5-HT (5-hydroxytryptamine), NA (noradrenaline), GABA (gamma-aminobutyric acid), CRF-R1 (corticotrophin releasing factor receptor subtype 1), EAAT2 (excitatory amino acid transporter 2), CCK (cholecystokinin), TRPV1 (transient receptor potential cation channel subfamily V member 1), and GFAP (glial fibrillary acidic protein). *Figure adapted from (Jennings et al., 2014).*

1.3.4 Neurotransmitters and neuromodulatory systems involved in hyperalgesia associated with negative affective state

Neurotransmitters, neuropeptides and other neuromodulators play a pivotal role in stress, negative affect and pain processing. Therefore, alterations in these neuromodulatory systems can influence hyperalgesia associated with negative affect.

1.3.4.1 Opioids

Analgesics targeting the endogenous opioid system are the most commonly prescribed (Trescot et al., 2008) and the opioid system is well recognised to modulate acute, inflammatory and neuropathic pain (Bushlin et al., 2010; Przewłocki and Przewłocka, 2001). This system is composed of the μ (mu)-, δ (delta)-, and κ (kappa)- opioid receptors. μ -, δ -and κ -opioid receptors are activated by the endogenous ligands such as β -endorphin, enkephalin and dynorphins, respectively. DuPen and colleagues have described the location of μ - and δ -opioid receptors and their expression at peripheral, spinal and supraspinal sites involved in pain processing (DuPen et al., 2007). Stress-induced mechanical hyperalgesia by whisker pad stimulation decreased when μ -opioid receptors in the RVM were blocked by with dermorphin-saporin (Reynolds et al., 2011). Administration of μ -opioid receptor antagonists (s.c. or i.p.), but not κ -opioid and δ -opioid receptor antagonists, blocked hyperalgesia in rats exposed to the FST (Le Roy et al., 2011; Suarez-Roca et al., 2006a, 2006b). These data suggest that only μ -opioid receptors play a role in hyperalgesia associated with negative affect. Under stress conditions, μ -opioid receptors can be desensitised by increased released β -endorphin (Le Roy et al., 2011, Suarez-Roca, et al., 2006b) and administration of antagonist blocks the desensitisation of the receptor, blocking the hyperalgesia associated with FST exposure.

The NOP receptor, before being confirmed as the fourth member of the opioid receptor family, was known as the opioid receptor-like 1 or ORL1. It is expressed in peripheral tissues (intestine, vas deferens, spleen and immune system) and also in the central nervous system (cortical brain areas, olfactory regions, hippocampus, amygdala and thalamus) (Tariq et al., 2013). A role for the NOP receptor has been demonstrated in peripherally mediated stress-induced hyperalgesia (Agostini et al., 2009) and further studies are required to decipher its role within the CNS in pain and stress interactions.

1.3.4.2 Glutamate and GABA

Glutamate is the main excitatory neurotransmitter in the brain and spinal cord and serves as an agonist at NMDA, AMPA/kainate or metabotropic glutamate receptors. Administration of ketamine, the NMDA receptor antagonist, blocked hyperalgesia in rats exposed to the FST (Suarez-Roca et al., 2006b). The NMDA receptor antagonist 2-amino-5-phosphonovaleric acid, when administered intrathecally, abolished hyperalgesia in a cold-stress model, but had no effect in the control rats (Okano et al.,

1995a, 1995b). The expression of glial glutamate transporter GLT1, the astrocytic marker GFAP, and the glutamate conversion enzyme glutamine synthetase, decreased in the spinal cord of rats expressing WAS-induced hyperalgesia, but the expression of the glial glutamate transporter, GLAST, was upregulated (Bradesi et al., 2011). Furthermore, visceral hyperalgesia was attenuated by NMDARs at the level of the spinal cord when pharmacologically blocked (Bradesi et al., 2011). At the spinal level, studies suggest the role of NMDA receptors in hyperalgesia mediated by negative affect.

GABA is the primary inhibitory neurotransmitter in the CNS. It acts on GABA_A (ligand-gated ion channel) and GABA_B (G-protein coupled) receptors (Bowerly and Smart, 2006). Microdialysis studies reveal that GABA release is reduced at the level of the spinal cord in forced swim stressed rats when exposed to a noxious stimulus (Quintero et al., 2011; Suarez-Roca et al., 2008), suggesting enhanced nociceptive transmission due to reduced GABAergic tone in the spinal cord. Similar findings have been reported in other studies of hyperalgesia associated with negative affective state (Eaton et al., 1999; Ibuki et al., 1996; Narita et al., 2011; Suarez-Roca et al., 2008). Systemic administration of diazepam (GABA_A receptor positive allosteric modulator) reduced hyperalgesia following exposure to the FST, and associated increases in *c-Fos* expression in the laminae I–VI of ipsilateral dorsal horn and administration of flumazenil (GABA_A negative allosteric modulator) blocked those effects (Suarez-Roca et al., 2008). All the above studies indicate that spinal GABAergic neurotransmission plays a role in modulating stress-induced hyperalgesia.

1.3.4.3 Cholecystokinin (CCK)

CCK is a sulphated octapeptide, abundant in the CNS as the CCK-8S isoform. There are two CCK receptor subtypes, CCK1 (CCKA) and CCK2 (CCKB), both of which are G-protein coupled receptors and expressed in the CNS (expression of CCK2 > CCK1). The CCK2 receptor is expressed highly in regions modulating pain and fear/emotional processing (Bowers et al., 2012; Chen et al., 2010; Kurrikoff et al., 2004; Li et al., 2013). In preclinical studies, deletion of the CCK2 receptor decreased hyperalgesia in a mouse neuropathic pain model (Kurrikoff et al., 2004), and ablation (CCK2 knockout mice) of the receptor increased μ - and δ -opioid receptors expression in the whole brain (Pommier et al., 2002). Intrathecal administration of the CCK2 receptor antagonist, L-

365,260, restored morphine's efficacy, after repeated exposure of rats to a stressor (Hawranko and Smith, 1999).

Anxiety-like behaviour was reported in male Wistar rats after intra-PAG injection of a CCK2 receptor agonist (Bertoglio and Zangrossi, 2005; Zanoveli et al., 2004). CCK stimulates descending facilitation of pain suggesting a pro-nociceptive role (Lovick, 2008). GABA_A receptor-mediated inhibitory postsynaptic currents in the dorsal root ganglia (Ma et al., 2006) and PAG (Mitchell et al., 2011) were inhibited upon administration of CCK-8S. Intra-RVM injection of a CCK2 receptor antagonist in a social defeat model of hyperalgesia associated with negative affect, prevented anxiety-like behaviour as assessed in the OF and EPM, and transient mechanical allodynia in the von Frey and the Randall–Selitto test, compared to vehicle-treated socially defeated rats. Microdialysis revealed that CCK in the frontal cortex was increased in social defeat rats. Pre-treatment with a CCK2 receptor antagonist, CI-998, prevented stress-induced increases in formalin-evoked nociceptive behaviour (Andre et al., 2005). Overall CCK is involved in hyperalgesia associated with negative affective state, but the exact mechanisms are yet to be elucidated.

1.3.4.4 Monoamines

Noradrenaline (NA) and 5-hydroxytryptamine (5-HT; serotonin), are known to play important roles in nociceptive transmission and descending inhibition, and in mediation and modulation of the stress response (Millan, 2002). The role of monoamines in negative affective state is well described. Systemic administration of 5-Hydroxytryptophan, a precursor of 5-HT, and L-DOPA, a precursor of dopamine, abolished repeated cold stress-induced hyperalgesia in mice (Ohara et al., 1991). Administration of fluoxetine, a SSRI, has been shown to attenuate chronic restraint stress-induced enhancement of formalin-induced nociceptive behaviour (Gameiro et al., 2006). Acute pre-treatment with tryptophan, a precursor of 5-HT, and chronic pre-treatment with clomipramine and fluoxetine blocked forced swim-induced hyperalgesia (Quintero et al., 2000). Vibration-induced mechanical hyperalgesia in rats was blocked by clonidine, an alpha-2-adrenoceptor agonist and inhibitor of the synaptic release of NA (Jørum, 1988) suggesting enhanced noradrenergic system activity is involved in mediating hyperalgesia associated with negative affect. Milnacipran, a dual 5-HT/NA uptake inhibitor (1-30mg/kg/i.p), reversed repeated forced-swim stress-induced muscle

hyperalgesia (Suarez-Roca et al., 2006a) suggesting the same effect. Similarly, in WKY rats, intracerebroventricular administration of a 5-HT_{2B} antagonist reduced pain behaviours during colorectal distension (O'Mahony et al., 2010) and 5-HT_{2B} receptors mediate restraint stress-induced visceral hypersensitivity in mice (Ohashi-Doi et al., 2010). Treatments using drugs regulating monoamines in hyperalgesia and negative effect are very well established but the underlying mechanism is yet to be determined.

1.3.4.5 The hypothalamic-pituitary-adrenal axis

The HPA axis plays a major role in the physiological response to stress (Herman and Cullinan, 1997) and pain (Bomholt et al., 2004). Clinical studies suggest that patients with IBS had lower baseline plasma levels of CRF but had increased or exaggerated levels of CRF upon stress exposure, which was also associated with enhanced visceral pain from colorectal distension (Posserud et al., 2004). The CRF receptor antagonist, alpha-helical CRF, reduced the abdominal pain evoked by electrical stimulation in IBS patients (Sagami et al., 2004). In preclinical models, systemic administration of a CRF1 receptor antagonist to rats inhibited WAS-induced visceral hyperalgesia (Million et al., 2003; Schwetz et al., 2004). In contrast, intracerebroventricular injection of a CRF1 antagonist had no effect on visceral hyperalgesia in rats (Larauche et al., 2009). In a rat model of neonatal stress, CRF1 receptors were shown to play a role involved in stress-induced visceral hyperalgesia (Schwetz et al., 2005). Abdelhamid et al reported the involvement of CRF2 receptors in musculoskeletal hyperalgesia in mice exposed to forced swim stress (Abdelhamid et al., 2013). Chronic subcutaneous administration of corticosterone to rats produced visceral hyperalgesia, as measured by colorectal distension (Hong et al., 2011). Furthermore, chronic water avoidance induces changes in transient receptor potential receptor TRPV1 and the cannabinoid (CB₁) receptor expression in the spinal cord (Hong et al., 2009, Hong et al., 2011) which were attenuated by the glucocorticoid receptor antagonist (RU-486). The results were consistent with previous work from Fereidoni et al where adrenalectomy diminished forced swim stress-induced hyperalgesia in rats (Fereidoni et al., 2007).

1.3.4.6 The sympathetic adrenomedullary and peripheral nervous systems

Peripheral components of stress response axes, in particular, the adrenergic system and other key mediators of pain processing in the periphery such as the DRG play a pivotal

role towards hyperalgesia associated with negative affect. Excitation of DRG neurons by stress hormones represents another potential mechanism underlying hyperalgesia associated with negative affect. Studies in cultured DRG neurons suggest that stress hormone signalling in colonic DRG neurons may play a role in sustained hyperexcitability of nociceptors (Ochoa-Cortes et al., 2014). In this study, overnight exposure of mouse DRG neurons to adrenaline and corticosterone-induced hyperexcitability in mouse DRG neurons, which was blocked by antagonists of the β 2-adrenoreceptor and glucocorticoid receptor, either individually or together. Rats exposed to the WAS model of hyperalgesia associated with negative affect showed differential effects of antagonist (β 2-adrenoreceptor and glucocorticoid receptor antagonists) treatment on visceromotor reflexes to colorectal distension, i.e. reduced visceromotor reflexes (Ochoa-Cortes et al., 2014). Khasar and colleagues reported that prolonged enhancement of bradykinin-induced hyperalgesia by unpredictable sound stress in rats requires a contribution from both the sympathoadrenal system and the HPA axis (Khasar et al., 2009). The contribution of the sympathoadrenal system to bradykinin-induced hyperalgesia was demonstrated by the absence of cutaneous and muscle hyperalgesia in unpredictable sound-stressed rats subjected to adrenal medullectomy (Khasar et al., 2009). Neonatal limited bedding (nesting material) for 1 week induced mild muscle hyperalgesia, which was inhibited following intrathecal treatment with antisense oligonucleotide (Alvarez et al., 2013). These studies suggest that hyperalgesia associated with negative affect is influenced by the sympathetic adrenomedullary and peripheral nervous systems.

1.4 TRPV1

1.4.1 Introduction

TRPV1 is a cation channel that belongs to the transient receptor potential (TRP) family, which is a diverse group of channels that contributes to a variety of physiological functions by regulating the entry of cations. There are 28 mammalian TRPs, divided into 6 subfamilies based on their structure: canonical (TRPC1-7), vanilloid (TRPV1-6), melastatin (TRPM1-8), ankyrin (TRPA1), polycystin (TRPP1-3) and mucolipin (TRPML1-3) (Montell, 2005; Pedersen et al., 2005). TRP channels are named after the role of the protein in the *Drosophila* phototransduction mutant responsible for transient response to bright light, as opposed to a sustained response (Montell, 2005). All

members of the six subfamilies are homologues and have in common a six transmembrane domain with a hydrophobic pore located between the fifth and sixth domains. They are located in the cell plasma membrane, endoplasmic reticulum (Caterina et al., 2000; Tominaga et al., 1998) and synaptic vesicles. TRP channels participate in divergent functions such as visual and auditory functions, speech, pain signal transduction, regulation of blood circulation, gut motility, mineral absorption and fluid balance, airway and bladder hypersensitivities, cell survival, growth and death (Inoue et al., 2006). TRP channels are activated by variety of stimuli including temperature, osmolarity, mechanical force, chemical stimulants and hence referred to as polymodal integrators (Caterina et al., 2000, 1997; Montell, 2005; Tominaga et al., 1998; Vander Stelt and Di Marzo, 2004; Vriens et al., 2009).

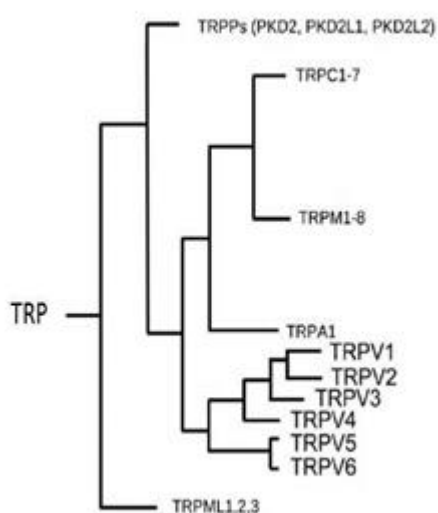


Figure 1.4: Phylogenetic classification of mammalian TRPs: transient receptor potential canonical (TRPC1-7), transient receptor potential vanilloid (TRPV1-6), transient receptor potential melastatin (TRPM1-8), transient receptor potential ankyrin (TRPA1), transient receptor potential polycystin (TRPP1-3) and transient receptor potential mucolipin (TRPML1,2,3) (Montell, 2005; Nilius et al., 2007; Vriens et al., 2009).

In 1997, the vanilloid receptor was cloned in rat cells from the DRG and demonstrated to be a subtype of non-selective cation channels related to the TRP family of ion channels (Caterina et al., 2000, 1997; Tominaga et al., 1998). This receptor was named by the authors as ‘vanilloid receptor subtype1’ (VR1). It was later renamed by the IUPHAR Nomenclature Committee as ‘Transient Receptor Potential Vanilloid Type 1’

as the receptor is activated by vanilloids, such as capsaicin, hence the ‘Vanilloid’ subtype term (Clapham, 2003).

1.4.2 Structure

Structural analysis reveals TRPV1 as a compact transmembrane region, formed by six transmembrane domains and intracellular N- and C-termini (Lishko et al., 2007; Moiseenkova-Bell et al., 2008). The N-terminal tail contains phosphorylation sites and ankyrin repeats which act as pockets for binding sites for calmodulin and ATP (Lishko et al., 2007). The C-terminal tail contains a TRP domain and binding sites for calmodulin, PIP₂, and endogenous TRPV1 inhibitor (García-Sanz et al., 2004; Numazaki et al., 2003; Ufret-Vincenty et al., 2011). The extracellular loop consists of amino acid residues Glu600 and Glu648 which are known for their physiological function such as pH sensitivity upon TRPV1 activation by protons (Jordt et al., 2000). Capsaicin mediates its action on the TRPV1 at the intracellular binding site (Jung et al., 1999). Multiple target sites have been identified by mutation and deletion studies which play a critical role in TRPV1 activation. Deletion of Thr550 in transmembrane region 4 reduced capsaicin sensitivity towards TRPV1 (Gavva et al., 2004). Deletion of Arg114 and Glu761 in the N- and C-termini blocked capsaicin-induced currents, having no effect on TRPV1 activation by heat (Jung et al., 2002). Mutations of Tyr511 and Ser512 blocked capsaicin responses, but activation by heat and protons had no effect (Jordt and Julius, 2002). TRPV1 upon activation forms homotetramers, but can also form functional oligomers with other TRP channels such as TRPA1 and TRPV3 (Moiseenkova-Bell et al., 2008; Staruschenko et al., 2010). The amino acids in the transmembrane domain and the C-terminus determine the affinity and specificity of subunit interactions by chimeric and deletion studies. There was no effect on the formation of tetramers when the N-terminus and C-terminus were replaced with TRPV4 in chimeric studies, suggesting the role of the transmembrane region in oligomerization (Hellwig et al., 2005). The C-terminus (684Glu-721Arg) on the TRPV1 domain is known to regulate the formation of functional tetramers, in the deletion studies (García-Martínez et al., 2000). TRPV1 is a cation channel, and its permeability to cations reduced upon substitution of Asp646 or Tyr671 in the pore domain when tested in the site-specific analysis (García-Martínez et al., 2000; Mohapatra et al., 2003). The permeability of TRPV1 to cations is dynamic, and factors such as the duration of the

stimulus and the agonist concentration play a vital role. Activation of TRPV1 regulates permeability and pore diameter to allow an influx of larger cations (Chung et al., 2008). SCAM (substituted cysteine accessibility method) has shown regulation in permeability of cations is mediated by amino acid residues (Tyr671 and Leu681 which gates for larger and smaller cations respectively) in transmembrane domain 6 (Salazar et al., 2009). Channel stimulation is also dependent on calcium permeability as activation by protons produces a smaller calcium current than activation by capsaicin (Samways et al., 2008). Refer to Figure 1.5

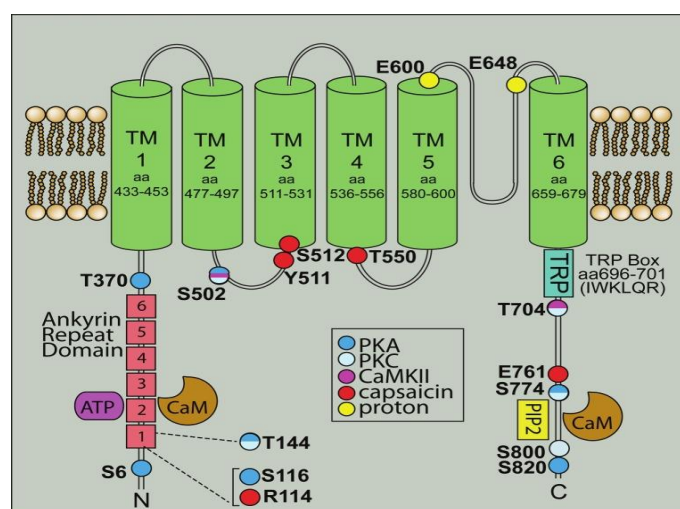


Figure 1.5: Structure of TRPV1. TRPV1 consists of six transmembrane domains and long intracellular N- and C- terminal tails. The fifth and sixth domain consist a pore region N-terminal consists of six ankyrin repeat domains allow binding of calmodulin and ATP which are essential in modulating TRPV1 activation. The C-terminus contains a TRP domain as well as binding sites for PIP2 and calmodulin. TRPV1 has multiple phosphorylation sites for PKA, PKC and CaMKII, in addition to putative sites for capsaicin and proton binding. TRPV1: transient receptor potential vanilloid 1, TM: transmembrane, aa: amino acid, CaM: calmodulin, ATP: adenosine triphosphate, PIP2: phosphoinositide 4,5-bisphosphate, PKA: protein kinase A, PKC: protein kinase C, CamKII: Ca²⁺/calmodulin dependent kinase II, Ligand binding site highlighted in red (Ho et al., 2012).

1.4.3 Localisation

TRPV1 is expressed highly in the DRG of C- and A δ - fibers. In these fibres, TRPV1 activation leads to increases in intracellular calcium levels which in turn induces the release of neuropeptides (calcitonin gene-related peptide and substance P) in the dorsal horn of the spinal cord (Price et al., 2005). Studies in rat and primate brain have shown that TRPV1 is widely expressed throughout the neuroaxis, including the cortex, hippocampus, basal ganglia, cerebellum and olfactory bulb, as well as in the mesencephalon and hindbrain (Mezey et al., 2000; Szabo et al., 2002) (Refer to table 1.2 for location of TRPV1 in various brain regions). TRPV1 in the PAG is located on

neurons, microglia or cytosol (Refer to table 1.3 for precise location of TRPV1 in the columns of PAG). Studies of the distribution of TRPV1 in the human brain have been more restricted, but a post-mortem study has shown that TRPV1 receptors have been found in the third and fifth layers of the human parietal cortex (Price et al., 2005). However, overall TRPV1 expression in the central nervous system (CNS) is considerably lower than in the DRG (Cavanaugh et al., 2011; Han et al., 2012; Sanchez et al., 2001; Szabo et al., 2002). Indeed, some studies have failed to detect the presence of TRPV1 in the CNS (Benninger et al., 2008; Caterina et al., 2000; Szallasi, 1995; Tominaga et al., 1998) possibly due to complexity in genes and strain-related variations (Sanchez et al., 2001; Sudbury et al., 2010). A sophisticated gene strategy where the TRPV1 gene was targeted by attaching two reporters, PLAP (placental alkaline phosphatase) and nlacZ (nuclear lacZ), onto the TRPV1 promoter gene and creating a specific line of mice (TRPV1^{PLAP-nlacZ}), was used to confirm TRPV1 expression in the CNS. This study reported that TRPV1 expression in the CNS is limited to certain brain regions and low when compared to expression in DRG (Cavanaugh et al., 2011). This restricted expression of TRPV1 in the CNS was confirmed by *in situ* hybridization experiments in rat, monkey and human brain (Cavanaugh et al., 2011). However, a number of recent pharmacological, genetic, radioligand binding and immunohistochemical studies suggest widespread distribution and functionality of TRPV1 across the CNS (Cavanaugh et al., 2011; Goswami et al., 2010; Han et al., 2013; O'Sullivan et al., 2000; Sanchez et al., 2001). TRPV1 is also found on non-neuronal cells such as keratinocytes (Southall et al., 2003), bladder urothelium (Lazzeri et al., 2004), smooth muscle (Birder et al., 2002), liver (Reilly et al., 2003), polymorphonuclear granulocytes (Heiner et al., 2003), pancreatic β -cells (Akiba et al., 2004), endothelial cells (Golech et al., 2004), lymphocytes (Saunders et al., 2007) and macrophages (Chen et al., 2003).

CNS region	Technique used	Reference
Telencephalon	[3]RTX autoradiography, ISH, RT-PCR, genetic-modified reporter (olfactory nuclei and entorhinal cortex only)	(Cavanaugh et al., 2011; Cristino et al., 2006; Mezey et al., 2000; Roberts et al., 2004; Tóth et al., 2005)
Hippocampal formation	[3]RTX autoradiography, IHC, ISH, RT-PCR, genetic-modified reporter	(Cavanaugh et al., 2011; Cristino et al., 2006; Mezey et al., 2000; Navarria et al., 2014; Roberts et al., 2004; Tóth et al., 2005)
Amygdala	[3]RTX autoradiography, ISH	(Cristino et al., 2006; Roberts et al., 2004)
Septal regions	[3]RTX autoradiography, ISH	(Cristino et al., 2006; Roberts et al., 2004)
Basal ganglia	[3]RTX autoradiography, IHC, ISH, RT-PCR	(Cristino et al., 2006; Roberts et al., 2004)
Thalamic nuclei	[3]RTX autoradiography, IHC, ISH	(Cristino et al., 2006; Roberts et al., 2004)
Hypothalamus	[3]RTX autoradiography, IHC, ISH, genetic-modified reporter	(Cavanaugh et al., 2011; Roberts et al., 2004)
Cerebellum	[3]RTX autoradiography, IHC	(Cristino et al., 2006; Roberts et al., 2004; Tóth et al., 2005)

Trigeminal ganglia	[3]RTX autoradiography, RT-PCR, ISH, genetic-modified reporter	(Cavanaugh et al., 2011; Cristino et al., 2006; Mezey et al., 2000; Roberts et al., 2004)
Midbrain and hindbrain regions	[3]RTX autoradiography, IHC, ISH, genetic-modified reporter	(Casarotto et al., 2012b; Cavanaugh et al., 2011; Cristino et al., 2006; McGaraughty et al., 2003; Mezey et al., 2000; Roberts et al., 2004; Starowicz et al., 2007; Tóth et al., 2005)

Table 1.2: Location of TRPV1 in the supraspinal regions. Table adapted from (Martins et al., 2014).

Location	Region	Technique Used	Species	Reference
Neurons	PAG	Reporter mice	Mice	(Cavanaugh et al., 2011)
Cytosol	DLPAG	Immunohistochemistry	Rats	(McGaraughty et al., 2003)
Cytosol	VLPAG	Immunohistochemistry	Rats	(McGaraughty et al., 2003)
Cell bodies/axons	PAG	Immunohistochemistry	Rats	(Cristino et al., 2006)
Cytoplasm, axons of neurons	VLPAG	Immunohistochemistry	Rats	(Maione et al., 2006)
VGAT and VGLUT neurons	VLPAG	Immunohistochemistry	Rats	(Starowicz et al., 2007)
Neurons	VLPAG	Electrophysiological recordings	Rats	(Liao et al., 2011)
Astrocytes and pericytes	PAG	Immunohistochemistry	Rats	(Tóth et al., 2005)
Neurons	DPAG	Immunohistochemistry	Rats	(Casarotto et al., 2012a)

Table 1.3: Location of TRPV1 on specific cells in the PAG

1.4.4 Pharmacology

As discussed earlier, TRPV1 is a molecular entity with diverse drug binding sites. Structure-activity studies have reported that capsaicin binds to an intracellular domain of the receptor (Fig. 1.5) (Gavva et al., 2004; Jordt and Julius, 2002), and the non-competitive antagonist ruthenium red binds at the extracellular vestibule of the pore domain (García-Martínez et al., 2000). The diverse binding nature of the compounds and their effects on downstream targets indirectly has led to the discovery of novel vanilloid-like agonists as well as other antagonists in order to improve the clinical efficacy of the TRPV1 compounds. Further summarized are the efforts undertaken to develop different classes of TRPV1 modulators.

1.4.3.1 TRPV1 Agonists

Compounds binding directly to the receptor are termed as agonists and compounds that do not bind to the receptor but indirectly affect the functioning of the receptor are known as sensitizers (Vriens et al., 2009).

1.4.3.1a Endogenous Agonists

Endovanilloids are capable of modulating the response of TRPV1 channels to thermal stimuli (van der Stelt and Di Marzo, 2004). TRPV1 activators are from the fatty acid pool and can be classified into conjugates of biogenic amines including N-arachidonylethanolamine (AEA), N-arachidonoyldopamine (NADA) (Appendino et al., 2008) or oxygenated eicosatetraenoic acids like the lipoxygenase products 12-, and 15-hydroperoxyeicosatetraenoic acids (12S-, 15S-HPETE) (Ahern, 2003; Hwang et al., 2000) or their reduced hydroxylic analogues such as prostaglandins, and leukotrienes (Huang et al., 2002). In addition to these, adenosine, ATP, polyamines have agonistic activity at TRPV1. Even acidic conditions such as pH 5.9 can activate TRPV1 which is common during inflammation (Ahern et al., 2006; Szallasi and Di Marzo, 2000; Xu et al., 2005). Endovanilloids of the fatty acid conjugate type resemble capsaicinoids as they have an aliphatic (AEA and OLEA) or aromatic (NADA) polar head and lipophilic moiety linked by an amide group (Hwang et al., 2000). Refer to table 1.4 for IC₅₀ values of endogenous agonists at TRPV1.

1.4.3.1b Exogenous Agonists of Natural, Semisynthetic, and Synthetic Origin

TRPV1 can be activated by various natural products such as piperine, eugenol, gingerol, resiniferatoxin (RTX) (Vriens et al., 2008). Capsaicin and its analogues also bind to the receptor and are an active ingredient from bell peppers (Caterina et al., 1997). Many other derivatives of capsaicin which are highly potent were produced but are hydrolytically unstable and act as prodrugs (Rosa et al., 2005). Refer to table 1.4 for IC₅₀ values.

The vanilloids show slow activation kinetics as their binding site is located in the intracellular portion of the receptor. Compounds such as capsaicinoids, resiniferonoids, and endovanilloids are highly lipophilic in nature and can cross the cell membrane to act on their intracellular binding site on TRPV1 (Appendino et al., 2002). Extracellular protons are believed to act primarily by increasing the probability of channel opening rather than by altering unitary conductance or interacting directly with the vanilloid-binding site (Baumann and Martenson, 2000). Acid solutions evoke ionic currents with an EC₅₀ at pH 5.4 when applied to outside-out but not inside-out membrane patches excised from HEK293 cells expressing TRPV1 (Baumann and Martenson, 2000).

1.4.3.1c Sensitizers

These compounds, usually inflammatory mediators, sensitize TRPV1 to chemical and physical stimuli. They might act via receptors of intrinsic tyrosine kinase pathways, G-protein-coupled receptors, or receptors coupled to the Janus tyrosine kinase/signal transducer and activator of transcription signalling pathway. TRPV1 is known to be phosphorylated by kinases including PKA (Bhave et al., 2002), protein kinase C (PKC; (Bhave et al., 2003), Ca²⁺/CaM-dependent kinase II (Jung et al., 2003), or Src kinase (Jin et al., 2004). TRPV1 activity is also strongly modulated by phosphatidylinositol phosphates (Nilius et al., 2004). PKA plays a pivotal role in the development of hyperalgesia and inflammation by inflammatory mediators (Mohapatra et al., 2003). PKC-dependent phosphorylation of TRPV1 occurs downstream from the activation of Gq-coupled receptors by several inflammatory mediators (Sugiura et al., 2002; Tominaga et al., 2001). The cyclin-dependent kinase Cdk5 plays an important role in pain transduction and nociceptive signalling (Pareek et al., 2006; Pareek and Kulkarni, 2006).

1.4.3.2 TRPV1 Antagonists

The elaborate class of antagonists is under immense attention because of the relevance of this protein for the management of chronic pain, migraine, and gastrointestinal disorders (Szallasi and Sheta, 2012). The antagonists are categorized into two major classes, classic antagonists are characterized by the presence of a carbonyl group and the non-classic antagonists where the carbonyl group is either absent or is unrecognisable.

1.4.3.2a Competitive Antagonists

Classic

Endogenous TRPV1 Antagonists are rare and not as abundant as TRPV1 agonists. Dynorphins, natural arginine-rich brain peptides that bind to κ -opioid receptors, are potent blockers of TRPV1 (Dessaint et al., 2004). More recently, the endogenous fatty acid amide hydrolase inhibitor N-arachidonyl serotonin has been shown to cause a direct block of TRPV1 and to inhibit the generation of

(Maione et al., 2007). Capsazepine was the first identified competitive antagonist for TRPV1, belongs to the class of 1,3-di(arylalkyl)thioureas, and is structurally related to capsaicin. Capsazepine competes for the capsaicin-binding site on TRPV1, inhibits capsaicin-mediated channel activation, and can displace RTX (Tominaga et al., 1998). 5-Iodoresiniferatoxin (5'-IRTX) belongs to the class of Iodinated Vanillyl derivatives. As RTX is an ultrapotent agonist for TRPV1, the introduction of an iodine atom ortho to the phenolic hydroxyl of the homovanillyl moiety reverts and generates the potent antagonist 5'-IRTX (Wahl et al., 2001).

Nonclassic

Two major structural types of nonclassic antagonists are imidazole derivatives and diaryl ethers and amines. Benzimidazole is the lead structure for imidazole derivatives and quinazoline for diaryl ethers and amines (refer to table 1.4 for examples)

1.4.3.2b Noncompetitive Antagonists (Pore Blockers)

Ruthenium red (RR) presumably interacts not only with the ligand binding site of TRPs but apparently also blocks its aqueous pore. RR binds TRPV1 with high potency in a

voltage-dependent manner (i.e., inward currents are efficiently blocked but not outward currents) (García-Martínez et al., 2000) Refer to table 1.4 for more examples.

Group	Compound	EC ₅₀ / IC ₅₀ (M)	Species	Reference
AGONISTS (EC₅₀)				
Endogenous agonists (fatty acids)	AEA	10 ⁻⁵	Rat	(Ahern, 2003)
	NADA	10 ⁻⁸ -10 ⁻⁷	Human and Rat	(Huang et al., 2002)
	12S-HPETE	10 ⁻⁵	Human and Rat	(Hwang et al., 2000)
Exogenous agonists of natural, semisynthetic and synthetic origin	Capsaicin	10 ⁻⁸ -10 ⁻⁶	Rodent	(Caterina et al., 1997)
	Piperine	10 ⁻⁵ -10 ⁻⁴	Human	(Liu and Simon, 1996)
	Eugenol	10 ⁻³ -10 ⁻²	Human	(Yang et al., 2003)
	Resiniferatoxin	10 ⁻¹⁰ -10 ⁻⁸	Rat	(Szallasi and Blumberg, 1989)
	olvanil	10 ⁻¹⁰ -10 ⁻⁹	Human	(Appendino et al., 2005)
	Phar	10 ⁻¹¹ -10 ⁻¹⁰	Human	(Appendino et al., 2005)

	Capmphor	10^{-3} - 10^{-2}	Rat	(Xu et al., 2005)
Antagonists (IC₅₀)				
Naturally occurring	Thapsigargin	10^{-6} - 10^{-5}	Rat	(Tóth et al., 2002)
	Yohimbine	10^{-5} - 10^{-4}	Rat	(Dessaint et al., 2004)
1,3-Di(arylalkyl)thioureas	Capsazepine	10^{-7} - 10^{-6}	Rat	(Caterina et al., 1997)
	JYL1421	10^{-8}	Rat	(Wang et al., 2002)
Iodinatede vanillyl derivatives	5-iodoRTX	10^{-9}	Rat	(Wahl et al., 2001)
Di(arylalkyl)- aryl(arylalkyl)ureas and	BCTC	10^{-9}	Rat	(Pomonis et al., 2003)
	A-425619	10^{-9}	Rat	(McDonald et al., 2008)
	SB-452533	10^{-8} - 10^{-7}	Human	(Rami et al., 2004)
	ABT-102	10^{-9}	Human	(Surowy et al., 2008)

Cinnamides	SB-366791	10^{-9}	Human, Rat	(Patwardhan et al., 2006)
	AMG-9810	10^{-8}	Human, Rat	(Elizabeth M. Doherty et al., 2004)
Carboxamides	SB-782443	10^{-8} - 10^{-7}	Human, Rat	(Westaway et al., 2008)
Non-classic antagonists Imidazole derivatives	AMG517	10^{-10}	Rat	(Gavva et al., 2007)
Non-classic antagonists Non-competitive	Ruthenium Red	10^{-7}	Rat	(García-Martínez et al., 2000)
	AG 489	10^{-8}	Rat	(Kitaguchi and Swartz, 2005)
	DD161515	10^{-7} - 10^{-6}	Rat	(García-Martínez et al., 2002)

Table 1.4: Agonists and antagonists for TRPV1—table adapted from (Vriens et al., 2009)

1.4.5 Physiology:

TRPV1 is expressed highly in the DRG of C-and A δ - fibers where its activation leads to increases in intracellular calcium levels which in turn releases neuropeptides (calcitonin gene-related peptide and substance P) (Price et al., 2005). Noxious heat (>43°C) can also activate TRPV1 indicating a role in thermal pain and hyperalgesia (Caterina et al., 1997). TRPV1^{-/-} mice display a reduced response to stimulation with TRPV1 agonists, thermosensation and hypoalgesia (Caterina et al., 2000). Inflammation has been linked to TRPV1-mediated nociception. It is known that intradermal injections of capsaicin can result in hyperalgesia in a dose-dependent manner (Simone et al., 1989). Oral administration of TRPV1 antagonists reduces capsaicin-induced hyperalgesia and mechanical allodynia in rodents (Cui et al., 2006). Many pro-inflammatory factors including substance P, nerve growth factor, bradykinin, prostaglandins and ATP can potentiate and sensitize TRPV1 (Zhang et al., 2007). The role of TRPV1 in pain and anxiety disorders is discussed following sections below. Apart from its role in pain processing, TRPV1 at the level of CNS is involved in synaptic transmission by regulating synaptic calcium levels and neurotransmitter release. In spinal cord slices from rats injected with Freund's complete adjuvant, the TRPV1 antagonist, SB-366791, produced a decrease in the frequency of excitatory post-synaptic currents (EPSCs) (Lappin et al., 2006). TRPV1-mediated increases in EPSC frequency have been observed in the substantia gelatinosa, PAG, medial preoptic nucleus, substantia nigra and locus coeruleus (Jiang et al., 2009; Karlsson et al., 2005; Marinelli et al., 2002; Xing and Li, 2007). Non-neuronal effects of TRPV1 are discussed following sections below in table 1.5.

Chapter 1

Location	Technique Used	Possible role/effects upon activation	Species	Reference
Arteriolar smooth muscle cells	RT-PCR, Ca ²⁺ imaging	vasoconstriction	Mouse,Rat	(Cavanaugh et al., 2011; Kark et al., 2008)
Mesenteric arteries and endothelial cells	IHC, RT-PCR, Ca ²⁺ imaging	vasorelaxation	Mouse	(Yang et al., 2010)
Laryngeal epithelium	IHC, RT-PCR, Ca ²⁺ imaging	Laryngeal nociceptors	Mouse	(Hamamoto et al., 2008)
Preadipocytes and adipose tissue	IHC, RT-PCR, Ca ²⁺ imaging	Adipogenesis	Mouse,Humans	(L. L. Zhang et al., 2007)
Urothelium	IHC, RT-PCR	Stretch-evoked ATP release	Mouse	(Birder et al., 2001)
Vascular smooth muscle	IHC, RT-PCR	vasoconstriction	Rat	(Yang et al., 2006)
Pancreatic B-cells	RT-PCR and western blot	Increased insulin secretion	Rat	(Akiba et al., 2004)
Corneal epithelium	western blot, Ca ²⁺ imaging	Inflammatory mediator secretion	Human	(F. Zhang et al., 2007)
Corneal endothelium	RT-PCR and western blot	Temperature sensation	Human	(Mergler et al., 2010)
Cerebromicrovascular endothelium	IHC, RT-PCR, Ca ²⁺ imaging	Regulation of blood-brain barrier permeability	Human	(Golech et al., 2004)

Blood	IHC	Nociception; role in inflammatory processes?	Human	(Saunders et al., 2007)
Epidermal keratinocytes	IHC, RT-PCR, Ca ²⁺ imaging	Noxious chemical sensor	Human	(Inoue et al., 2002)
Synoviocytes	RT-PCR, Ca ²⁺ imaging	Adaptive/pathological changes in arthritic inflammation	Human	(Kochukov et al., 2006)
Nasal vascular endothelium, epithelial and submucosal glands	IHC, RT-PCR	Stimulate epithelial secretions	Human	(Seki et al., 2006)

Table 1.5: Physiological functions of TRPV1 in peripheral non-neuronal cells. IHC: Immunohistochemical analysis, RT-PCR: real time-polymerase chain reaction. Table adapted from (Fernandes et al., 2012).

1.5. Role of TRPV1 in pain

1.5.1. Acute pain

The periaqueductal grey - rostral ventromedial medulla (PAG-RVM) pathway is very important in pain processing and modulation. PAG-mediated antinociception involves the recruitment of pain-modulating RVM neurons via the descending pain pathway (Bajic and Proudfit, 1999). Capsaicin, when injected into the dorsolateral periaqueductal grey (DLPAG), increased the latency of nociceptive responding to noxious heat, indicating that stimulation of TRPV1 within the descending inhibitory pain pathway can cause antinociception (Palazzo et al., 2002). Microinjection of capsaicin into the ventrolateral periaqueductal grey (VLPAG) increased the threshold of thermal pain sensitivity in rats (Starowicz et al., 2007). Opposite effects were found with 5'-IRTX, a selective TRPV1 antagonist that facilitated nociceptive responses and, at an inactive dose, abolished capsaicin-mediated antinociception, implying that the effect of capsaicin is mediated by TRPV1 in the VLPAG (Maione et al., 2006). The antinociceptive effect of intra-VLPAG capsaicin was accompanied by an increase in glutamate release in the RVM as measured by *in vivo* microdialysis, which was also blocked by a *per se* inactive dose of I-RTX. The TRPV1 antagonist itself reduced the release of glutamate, thus suggesting that VLPAG TRPV1 tonically stimulates glutamatergic output to the RVM with a concomitant inhibition of nociception (Starowicz et al., 2007). Hyperalgesia or analgesia have been observed following intra-VLPAG administration of the fatty acid amide hydrolase inhibitor (FAAH) URB597 depending on whether VLPAG cannabinoid receptors or TRPV1 have been activated (Maione et al., 2006). It was proposed that AEA-mediated activation of TRPV1 leads to analgesia, while hyperalgesia may be due to increases in VLPAG 2-arachidonoylglycerol (2-AG) leading to CB₁ receptor stimulation which in turn leads to inhibition of the antinociceptive PAG-RVM descending pathway (Maione et al., 2006).

The ON and OFF neurons in the RVM have been shown to respond to capsaicin administered into the PAG (Maione et al., 2006; McGaraughty et al., 2003; Starowicz et al., 2007). Intra-DLPAG microinjection of capsaicin is followed by a decrease in the tail flick-related ON cell burst activity and an increase in the tail flick latency (McGaraughty et al., 2003). Later on, due to desensitization of the receptor (due to prolonged exposure to capsaicin), antinociception correlating with increased OFF cell activity was reported (McGaraughty et al., 2003). Similarly, Starowicz et al. (2007)

have shown that intra-VLPAG administration of capsaicin caused a decrease in the firing activity of RVM ON-cells, and an increase in the firing of the OFF-cells (Starowicz et al., 2007). Moreover, microinjections of capsaicin into the VLPAG have also been shown to increase withdrawal latency in the rat hot-plate test, with evidence that activation of TRPV1 in the VLPAG induces antinociception via mGlu receptor-mediated 2-AG retrograde signalling in the RVM (Liao et al., 2011). Intra-VLPAG administration of the FAAH inhibitor, URB597 which is known to enhance endogenous AEA levels, stimulated OFF cell activity in the RVM and inhibited ON cell activity (Maione et al., 2006). This effect on RVM activity was abolished by intra-VLPAG administration of the TRPV1 antagonist capsazepine, suggesting that FAAH substrates (likely AEA) activate TRPV1 on VLPAG neurons, with projections from the PAG to the RVM mediating the subsequent stimulation of RVM OFF-cells and inhibition of ON cells. De Novellis et al. administered N-arachidonoyl-serotonin (AA-5-HT), a compound with a dual ability to inhibit FAAH and block TRPV1, into the VLPAG, and measured endocannabinoid levels, RVM ON and OFF cell activities, thermal nociception in the tail flick test and formalin-induced nociceptive behaviour (de Novellis et al., 2008). They found that AA-5-HT increased AEA levels in the VLPAG and had antinociceptive effects in both the tail-flick and formalin tests. Moreover, intra-VLPAG administration of AA-5-HT depressed the activity of both OFF cell and ON cells in the RVM. These effects of AA-5-HT were similar to those seen following co-administration of the FAAH inhibitor URB597 and the selective TRPV1 antagonist 5'-IRTX into the VLPAG (de Novellis et al., 2008). The FAAH substrate, palmitoylethanolamide (PEA), when microinjected into the VLPAG of rats, was antinociceptive in the tail-flick test, concomitantly decreasing the ongoing activity of the OFF cells in the RVM and increasing the latency of tail flick-evoked onset of ON cell activity (de Novellis et al., 2012). These latter effects on RVM cell activity were blocked by the TRPV1 antagonist I-RTX, suggesting that TRPV1 modulates PEA-induced effects within the PAG-RVM circuitry (de Novellis et al., 2012). PEA does not directly bind to TRPV1, but through the substrate competition at FAAH it can indirectly elevate AEA levels, which in turn may bind to TRPV1 and induce antinociception. Recently Kerckhove et al. have elucidated the role of TRPV1 in modulating the antinociceptive effect of paracetamol in mice via Ca(v)3.2 channels. Analgesic effect of TRPV1 is known to be mediated by T-type calcium channels (Ca(v)3.2), as AEA is known to elicit analgesic response by blocking the Ca(v)3.2 (Barbara et al., 2009). In this study by Kerckhove and colleagues, activation of TRPV1 induced a strong

inhibition of Ca(v)3.2 current and i.c.v. administration of AM404 or capsaicin lead to antinociception that is lost in Ca(v)3.2^(-/-) mice (Kerckhove et al., 2014). Refer to figure 1.6 for the proposed mechanism of paracetamol in the brain.

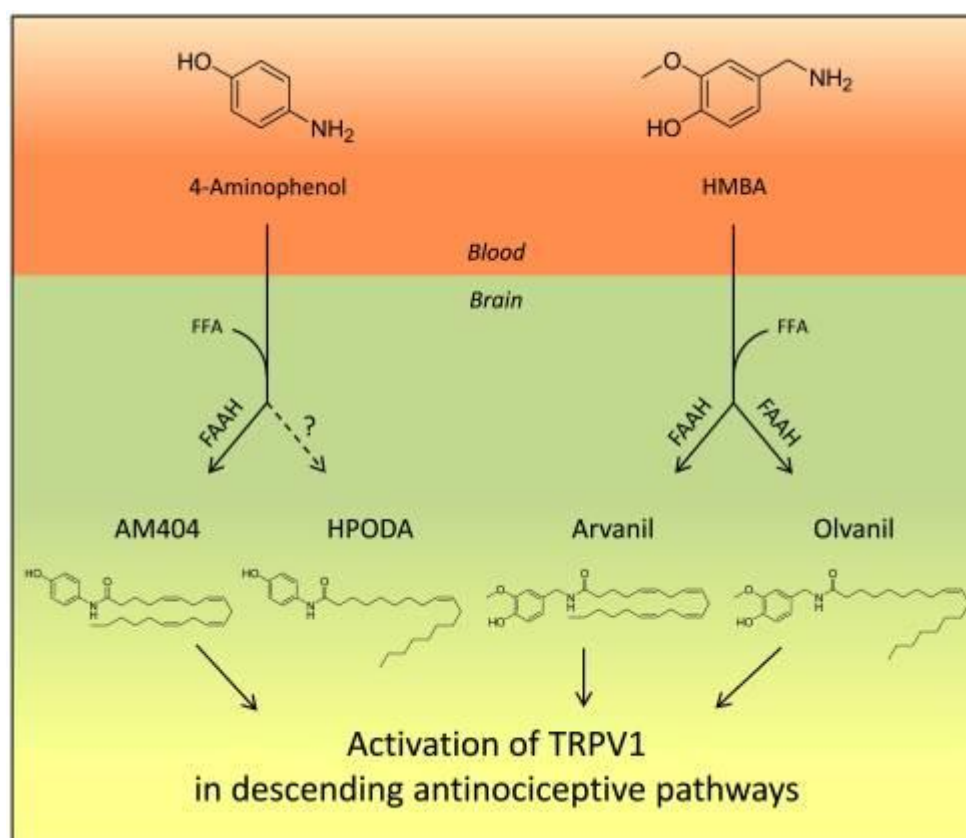


Figure 1.6: Mechanism for de-acetylated paracetamol metabolites 4-aminophenol and 4-hydroxy-3-methoxybenzylamine (HMBA) metabolism and antinociceptive activity at TRPV1 in the brain (Barrière et al., 2013). AM404: N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide, HPODA: N-(4-hydroxyphenyl)-9Z-octadecenamide, FFA:free fatty acids. (Barrière et al., 2013)

1.5.2. Chronic pain

Intracerebroventricular (i.c.v.) administration of the TRPV1 receptor antagonist, (1-[3-(trifluoromethyl)pyridin-2-yl]-N-[4-(trifluoromethyl sulfonyl)phenyl]-1,2,3,6-tetrahydropyridine-4-carboxamide, A-784168, significantly reduced weight bearing in the sodium monoiodoacetate model of osteoarthritis and reduced CFA-induced chronic inflammatory thermal hyperalgesia, suggesting that TRPV1 in the brain plays a key role in chronic inflammatory pain (Cui et al., 2006). Furthermore, i.c.v. administration of the plant-derived alkaloid (-)-cassine prevented mechanical hyperalgesia induced by carrageenan, an effect mediated by TRPV1 receptors (da Silva et al., 2012). Site-specific delivery of the dual TRPV1 and FAAH blocker, AA-5-HT, into the prelimbic-infralimbic cortex significantly decreased mechanical allodynia in the

SNI model of neuropathic pain in mice (Giordano et al., 2012). Similarly, intra-cortical (prelimbic-infralimbic cortex) administration of AA-5-HT was more effective in reducing SNI-induced mechanical allodynia than I-RTX (de Novellis et al., 2011). Robust evidence suggests that TRPV1 mediates visceral hyperalgesia in water avoidance test via the endocannabinoid pathway (Hong et al., 2009b). Thus, while fewer studies have investigated the role of supraspinal TRPV1 in animal models of chronic pain versus acute pain, these studies together suggest the involvement of supraspinal TRPV1 in the development and/or maintenance of both chronic inflammatory and neuropathic pain. Table 1.6 provides a summary of studies to date that have investigated the effects of intracerebral administration of TRPV1 ligands on nociceptive behaviour in animal models of acute and chronic pain.

The small number of studies that have specifically investigated the role of TRPV1 in SIH will be reviewed in the Introduction to Chapter 2 (Section 2.1).

Drug and Dose	Agonist OR Antagonist	Route of admin /target region in CNS	Test/Effect on behaviour	Species	Reference
Capsaicin(1–3–6 nmol)/rat	Agonist	i.c.(DLPAG)	Antinociceptive effect/thermosensitivity test	Rat	(Palazzo et al., 2002)
Capsazepine (6nmol/rat)	Antagonist	i.c.(DLPAG)	Blocked the capsaicin-induced antinociception/thermosensitivity test	Rat	(Palazzo et al., 2002)
Capsaicin (6nmol/rat)	Agonist	i.c.(VLPAG)	Antinociceptive effect/thermosensitivity test	Rat	(Maione et al., 2006)
Capsaicin (3 and 6 nmol/rat)	Agonist	i.c.(VLPAG)	Antinociceptive effect/RVM extracellular recordings and tail flick test	Rat	(Starowicz et al., 2007)
I-RTX (0.5 and 1nmol/rat)	Antagonist	i.c.(VLPAG)	Blocked the antinociceptive effect of capsaicin/RVM extracellular recordings and tail flick test	Rat	(Starowicz et al., 2007)
Capsaicin (10 nmol/rat)	Agonist	i.c.(dPAG)	Initially produced hyperalgesia followed by analgesia/RVM extracellular recordings and tail flick test	Rat	(McGaraughty et al., 2003)

			flick test		
Capsazepine (10nmol/rat)	Antagonist	i.c.(dPAG)	Blocked the hyperalgesic effect of capsaicin/RVM extracellular recordings and tail flick test	Rat	(McGaraughty et al., 2003)
Capsaicin (0, 0.01, 0.1 or 1.0nmol /0.2µl)/mice	Agonist	i.c.(dPAG)	Capsaicin (only 1nmol/0.2µl) decreased the time spent licking the formalin-injected paw/Formalin test	Mice	(Mascarenhas et al., 2015)
Capsazepine (0, 10 or 30 nmol/0.2µL)/mice	Antagonist	i.c.(dPAG)	No effect on time spent licking the formalin-injected paw/Formalin test	Mice	(Mascarenhas et al., 2015)
Capsaicin (1nmol /0.2µL) + Capsazepine (30nmol/0.2µL)/mice	Agonist + antagonist	i.c.(dPAG)	Capsazepine blocked antinociceptive behaviour induced by capsaicin/ Formalin test	Mice	(Mascarenhas et al., 2015)
Capsaicin (6nmol/rat)	Agonist	i.c.(VLPAG)	Antinociceptive effect/hot-plate test	Rat	(Liao et al., 2011)
SB 366791 (50 nmol)/rat	Antagonist	i.c.(VLPAG)	Abolished Antinociceptive effect/hot-plate test	Rat	(Liao et al., 2011)
AA-5-HT (0.1–0.2–	Antagonist	i.c.(VLPAG)	Pro-nociceptive at lower doses	Rat	(de Novellis et

0.5nmol)/rat			and antinociceptive at higher doses/RVM extracellular recordings and tail flick test		al., 2008)
I-RTX (0.5nmol/rat)	Antagonist	i.c.(VLPAG)	Blocked the AA-5-HT effects /RVM extracellular recordings and tail flick test	Rat	(de Novellis et al., 2008)
PEA (3 or 6nmol)/rat	N/A	i.c.(VLPAG)	Antinociceptive effect /RVM extracellular recordings and tail flick test	Rat	(de Novellis et al., 2012)
I-RTX (1nmol/rat)	Antagonist	i.c.(VLPAG)	Blocked the PEA-induced effects /RVM extracellular recordings and tail flick test	Rat	(de Novellis et al., 2012)
Capsaicin(30mM /rat)	Agonist	i.c.(RVM)	Reduced the inflammatory pain response in Streptozocin-induced diabetic rats during phase 2 of formalin test	Rat	(Silva et al., 2016)
Capsaicin (10µg/ mice)	Agonist	i.c.v	Antinociception /formalin test and von Frey test	Cav3.2 ^{+/+} mice	(Kerckhove et al., 2014)
Capsaicin (10µg/mice)	Agonist	i.c.v	No-effect/formalin test and von Frey test	Cav3.2 ^{-/-} mice	(Kerckhove et al., 2014)

Capsazepine (100nmol/mice)	Antagonist	i.c.v.	Blocked antinociceptive effects of 4-aminophenol and HMBA /Formalin test	Mice	(Barrière et al., 2013)
A-784168(100nmol)	Antagonist	i.c.v	Reduced weight bearing/Sodium monoiodoacetate model of osteoarthritis	Rat	(Cui et al., 2006)
A-784168(100nmol)	Antagonist	i.c.v	Reduced CFA-induced chronic inflammatory thermal hyperalgesia/thermal test	Rat	(Cui et al., 2006)
Cassine (10µg)	Agonist	i.c.v	Blocked mechanical hyperalgesia/ carrageenan model	Rat	(da Silva et al., 2012)
AA-5-HT (0.1-0.25-1 nmol), I-RTX (0.25-0.5-1nmol)	Antagonist	Intra-cortical(PL-IL)	reducing mechanical allodynia /SNI model	Rat	(de Novellis et al., 2011; Giordano et al., 2012)

Table 1.6: Effects of pharmacological modulation of supraspinal TRPV1 in animal models of pain. i.c.: intracerebral, i.c.v: intracerebroventricular, DLPAG: dorsolateral periaqueductal gray, dPAG: dorsal periaqueductal gray, AA-5HT: N-arachidonoyl-serotonin, SB366791: N-(3-Methoxyphenyl)-4-chlorocinnamide, A-784168:1-[3 - (trifluoromethyl)pyridin-2-yl]-N-[4-(trifluoromethyl sulfonyl)phenyl]-1,2, 3,6-tetrahydropyridine-4-carboxamide, PL-IL:Prelimbic-Infralimbic cortex, SNI:Spared nerve injury, N/A-not applicable.

1.6 Role of TRPV1 in negative affective state

1.6.1 Anxiety

1.6.1.1 Generalized Anxiety Disorder (GAD)

The pharmacological studies summarised in Table 1.7 indicate that TRPV1 in the dorsal PAG (dPAG), the hippocampus (HPC), mPFC and the BLA modulates anxiety-related behaviour in the rat/mouse EPM. Systemic injection of TRPV1 antagonists (capsazepine and SB-366791) or agonist (Olvanil) has been shown to produce anxiolytic or anxiogenic effects, respectively, in the EPM (Kasckow et al., 2004; Micale et al., 2009). TRPV1 KO mice exhibit an anxiolytic phenotype, suggesting that TRPV1 plays a role in anxiety-related behaviour (Marsch et al., 2007). Intra-ventromedial prefrontal cortex (vmPFC) injections of the TRPV1 antagonist capsazepine had an anxiolytic effect in the EPM and Vogel conflict (VCT) tests and attenuated the expression of contextual fear conditioning in rats (Aguiar et al., 2009; Rubino et al., 2008; Terzian et al., 2014). The role of TRPV1 was confirmed by administration of the TRPV1 antagonists 6-iodo-nordihydrocapsaicin and capsazepine (Rubino et al., 2008; Terzian et al., 2014). Similar to the vmPFC, blockade of TRPV1 in the vHPC and DLPAG also elicited anxiolytic effects in the EPM (Santos et al., 2008; Terzian et al., 2009). Recent studies proved that TRPV1 and NMDA receptors are essential in AEA-mediated anxiety-like responses (Fogaça et al., 2013). High doses of AEA, ineffective doses of TRPV1 antagonist (capsazepine) or NMDA antagonist (AP7) failed to produce any changes in EPM alone, but the administration of capsazepine or AP7 prior to the higher dose of AEA produced an anxiolytic effect (Fogaça et al., 2013). Studies from the same group have reported that lack of anxiogenic effects of higher doses of AEA is due to NO (nitric oxide) synthesis (Batista et al., 2015). A NO scavenger (c-PTIO) restored the anxiolytic effect of a high dose of AEA in the EPM and VCT (Batista et al., 2015).

1.6.1.2 Panic Disorder

The DLPAG is known for its role in coordinating freezing, fight and flight behaviors in threatening situations, such as the presence of a predator (Mascarenhas et al., 2013).

Evidence suggests that electrical stimulation of the DLPAG in humans induces symptoms similar to a panic attack (Schenberg et al., 2001). Corroborating this finding, neuroimaging studies show increased DPAG activity in panic patients (Del-Ben et al., 2009) or in healthy volunteers exposed to a proximal threatening stimulus such as predator exposure (Mobbs et al., 2007). Panic-like responses can be modulated by several neurotransmitters including serotonin, GABA, glutamate and nitric oxide (Moreira and Guimaraies, 2004). Several studies indicate the presence of TRPV1 in the PAG [Refer to section 1.4.2] which may influence the panic response. Local injection of the TRPV1 antagonists capsazepine or SB366791 into the dPAG attenuated panic-like behavior induced by electrical stimulation (Terzian et al., 2009). TRPV1 antagonism in the DLPAG had similar effects in three other animal models of panic induced by [i] local injection of the excitatory amino acid N-methyl-d-aspartate [NMDA], [ii] local injection of the nitric oxide donor SIN-1, and [iii] exposure to the open arms of the elevated T-maze (Almeida-Santos et al., 2013; Lisboa and Guimarães, 2012a). These results suggest that TRPV1 in the DLPAG facilitates defensive responses in threatening situations.

1.6.1.3 Obsessive-compulsive disorder (OCD)

Marble-burying behaviour (MBB) is a commonly used model for assessing compulsive activity in rodents (Broekkamp et al., 1986). Studies indicate that the burying behaviour in rodents is an unconditioned, species-specific defensive reaction which is not associated with physical danger, and to which animals do not habituate upon repeated testing. Thus, MBB models some of the clinical symptoms of obsessive-compulsive disorder (OCD) which is characterized by recurrent obsessions or compulsions that severely impair daily routine.

A recent study by Umathe et al. (2012) investigated the effects of capsaicin and capsazepine, administered i.c.v., on MBB. This study revealed that capsaicin produced compulsive effects (increased marble buying), similar to those of high-dose AEA, whereas capsazepine dose-dependently decreased the burying behaviour (Umathe et al., 2012). These observations support the hypothesis that central TRPV1 might mediate the pro-compulsive effect of high doses of AEA. Central administration of lower doses of AEA, or drugs that elevate levels of AEA (AM404/URB597), inhibited MBB, suggesting anti-compulsive effects (Umathe et al., 2012). Pre-treatment with a CB₁

receptor antagonist (i.c.v.) abolished the anti-compulsive effect of AEA whereas the TRPV1 antagonist capsazepine blocked the procompulsive effect of higher doses of AEA (Umathe et al., 2012). Therefore these results suggest that TRPV1 activation leads to an increase in OCD-like behaviour, with blockade of TRPV1 alleviating such behaviour. Further research employing additional animal models is required to determine the precise role of central TRPV1 in the regulation of compulsive behaviour. Overall, preclinical studies show that TRPV1 plays a key role in anxiety-related behaviour. Given the high degree of co-morbidity between anxiety disorders and chronic pain, and overlap in the TRPV1-expressing neuroanatomical substrates involved in both anxiety and pain, it is likely that TRPV1 also plays an important role in anxiety-pain interactions and further research in this area is warranted. Figure 1.7 represents a synthesis of the pain and anxiety literature reviewed above.

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Drug and Dose	Agonist or Antagonist	Route of admin /target region in CNS	Test/Effect on behaviour	Species	Reference
Anxiety					
Olvanil 0.2-5.0 mg/kg;	Agonist	i.p	EPM/Anxiogenic	Rat	(Kasckow et al., 2004)
Capsazepine (1–10 µg/kg)	Antagonist	i.p.	EPM/Anxiolytic	Rat	(Kasckow et al., 2004)
SB366791 (0.1–2.5 mg/kg); olvanil (0.1 mg/kg);AA-5-HT (0.1–5 mg/kg)	Antagonist; Agonist; Antagonist	i.p.	EPM/Anxiolytic	Mouse	(Micale et al., 2009)
Capsazepine (1, 10 ,30 and 60 nmol)	Antagonist	i.c. (mPFC)	EPM and VCT/Anxiolytic	Rat	(Aguiar et al., 2009)
Methanamide 0.1-10 µg Capsaicin 1-10µg and 1nmol	Antagonist Agonist	i.c (mPFC)	EPM /Anxiogenic	Rat	(Rubino et al., 2008)

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AA-5-HT (0.25–0.5 nmol)	Antagonist	i.c. (BLA)	EPM /Anxiolytic	Rat	(John and Currie, 2012)
AMG 9810 (0.003, 0.03 and 0.3 µg)	Antagonist	i.c. (dHPC)	EPM /Anxiolytic	Rat	(Hakimizadeh et al., 2012)
Capsaicin (0.003, 0.03 and 0.3 µg)	Agonist	i.c. (dHPC)	EPM /Anxiogenic	Rat	(Hakimizadeh et al., 2012)
Capsazepine (0.2–2 nmol)	Antagonist	i.c. (vHPC)	EPM /Anxiolytic	Rat	(Santos et al., 2008)
Capsaicin (0.01, 0.1 and 1 nmol)	Agonist	i.c. (dPAG)	EPM /Anxiogenic	Mouse	(Mascarenhas et al., 2013)
Capsaicin (0.01, 0.1 and 1 nmol)	Agonist	i.c. (DLPAG)	EPM and VCT /Anxiolytic	Rat	(Terzian et al., 2009)
6-iodonordihydrocapsaicin (3 nmol) and c-PTIO (0.3 nmol)	Antagonist	i.c (DLPAG)	EPM and VCT /Anxiolytic	Rat	(Batista PA, Fogaça MV, 2015)
c-PTIO (0.3 nmol) and AEA (50 ,200 pmol)		i.c (DLPAG)	EPM and VCT /Anxiolytic	Rat	(Batista PA, Fogaça MV, 2015)
Capsazepine (10nmol) and AEA (50 and 200 nmol)	Antagonist	i.c (DLPAG)	EPM and VCT /Anxiolytic	Rat	(Fogaça et al., 2013)

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Capsazepine (1, 10 ,30 and 60 nmol)and 6-iodonordihydrocapsaicin (3 nmol)	Antagonist	i.c. (mPFC)	Conditioned fear/Decrease in fear-related behaviour	Rat	(Terzian et al., 2014)
Capsaicin 1-10µg and 1nmol	Agonist	i.c (mPFC)	Conditioned fear/Increase in fear-related behaviour	Rat	(Terzian et al., 2014)
Capsazepine (60 nmol/0.2 µl)	Antagonist	i.c. (DLPAG)	Predator exposure /decrease in fear-related behaviour	Rat	(Aguiar et al., 2015)
Capsazepine (1-60 nmol) 30nmol*	Antagonist	i.c. (DLPAG)	ETM / panicolytic-like effects	Rat	(Almeida-Santos et al., 2013)
SB366791(10nmol) Capsazepine (0.1, 1 and 10 nmol)	Antagonist	i.c.(DLPAG)	Escape threshold determination/panicolytic-like effects	Rat	(Casarotto et al., 2012a)
Capsazepine (30 nmol)	Antagonist	i.c.(DLPAG)	Escape threshold determination/panicolytic-like effects	Rat	(Lisboa and Guimarães, 2012a)
Capsazepine (100 µg)	Antagonist	i.c.v	Abolished marble-burying behaviour	Mice	(Umathe et al., 2012)
Depression					
Olvanil 0.2-5.0 mg/kg	Agonist	i.p	Antidepressant effect/ Porsolt swimming test	Rat	(Kasckow et al., 2004)

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Combination of capsazepine (50 µg/mouse) with α -spinsteroil (1, 2mg/kg)	Antagonist	i.c.v and i.p	Reduction in the total immobility (Antidepressant effect) / forced swimming test	Mice	(Socała and Wlaź, 2016)
Capsaicin (200 and 300 µg/mouse) and capsazepine (100 and 200 µg/mouse)	Agonist+ Antagonist	i.c.v	Antidepressant effect/ forced swimming test	Mice	(Manna and Umathe, 2012)
capsaicin (0.1, 1 and 2.5 mg/kg) and olvanil (0.1, 1 and 5 mg/kg)	Agonist	i.p	Antidepressant effect/ forced swimming test	Mice	(Hayase, 2011)
RTX (0.25 µg/kg i.t.)	Agonist	i.t	Increased immobility (depressive -like) /forced swimming test	Rat	(Abdelhamid et al., 2014)
Amitriptyline (10 mg/kg)and Ketamine (50 mg/kg)	N/A	i.p	Decreased the immobility caused by RTX (Antidepressant effect) / forced swimming test	Rat	(Abdelhamid et al., 2014)
AA-5-HT (2.5 and 5 mg/kg)	Antagonist	i.p	Reversal of behavioral despair (Antidepressant effect) / forced swimming test	Rat	(Navarria et al., 2014)

Table 1.7: Effects of pharmacological modulation of TRPV1 in animal models of anxiety and depression. i.p. : intraperitoneal, i.t : intrathecal, i.c.: intracerebral, i.c.v: intracerebroventricular, DLPAG: dorsolateral periaqueductal gray, dPAG: dorsal periaqueductal gray, ETM: elevated T-maze, EPM: elevated plus-maze, VCT: Vogel Conflict test, mPFC: medial prefrontal cortex, vHPC: ventral hippocampus, dHPC: dorsal hippocampus, BLA: basolateral amygdala, AA-5HT: N-arachidonoyl-serotonin, SB366791: N-(3-Methoxyphenyl)-4-chlorocinnamide, RTX: resiniferatoxin. N/A: Not applicable.

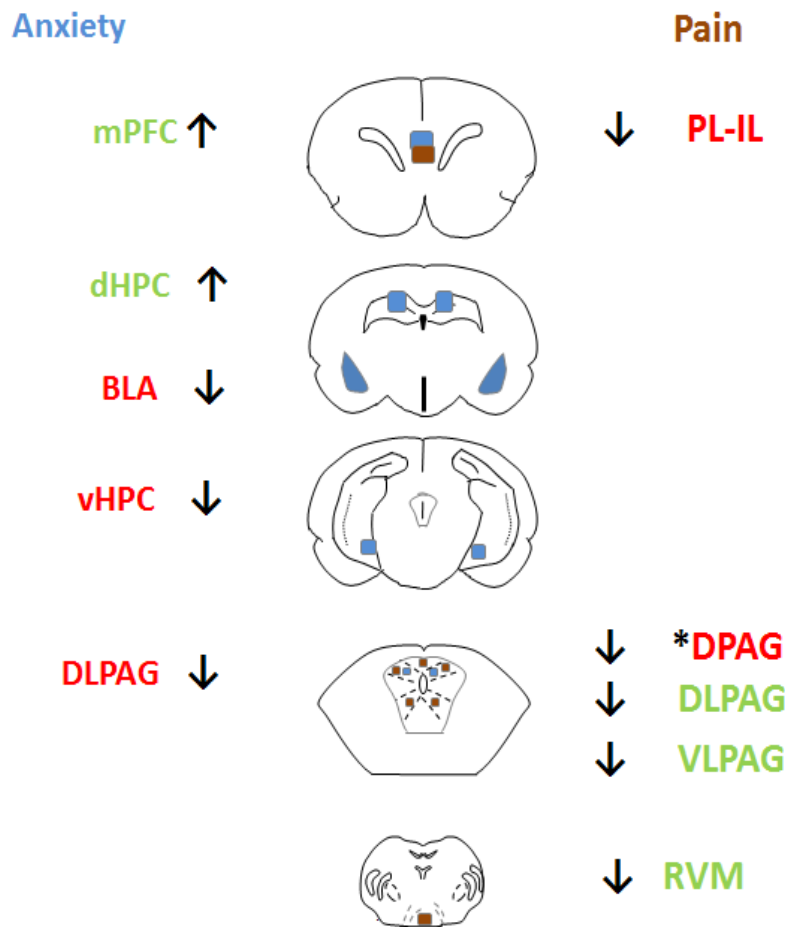


Figure 1.7: A synthesis of the literature reviewed herein on the role of TRPV1 in discrete brain regions in pain- and anxiety-related behaviour. Green coloured text indicates the activation of TRPV1 in that brain region and red colour indicates the blockade/desensitization of the receptor in that brain region. ↑ denotes an increase in anxiety/pain related behaviour and ↓ denotes a decrease in anxiety/pain-related behaviour. * denotes initial activation of the receptor followed by desensitisation. Blue shading of a brain region denotes anxiety-related studies of TRPV1 in that region and brown shading denotes pain-related studies of TRPV1 in that region. DLPAG: dorsolateral periaqueductal gray, dPAG: dorsal periaqueductal gray, mPFC: medial prefrontal cortex, vHPC: ventral hippocampus, dHPC: dorsal hippocampus, BLA: basolateral amygdala, RVM: rostral ventromedial medulla, PL-IL: prelimbic-infralimbic cortex.

1.6.2 Depression

TRPV1 antagonists, administered systemically, have been shown to produce antidepressant-like effects in both rats and mice, suggesting a role for TRPV1 in depression (Hayase, 2011; Kasckow et al., 2004; Kulisch and Albrecht, 2013; Manna and Umathe, 2012; You et al., 2012) (table 1.7 above). In the forced swim test, TRPV1 KO mice displayed lower immobility when compared to wild-type mice, indicating less

behavioural despair in mice lacking TRPV1 (You et al., 2012). Similarly, systemic administration of the TRPV1 antagonist capsazepine decreased the immobility time in a dose-dependent manner in the forced swim test (Hayase, 2011). Another paradigm for assessment of antidepressant-like activity is novelty-suppressed feeding. In this test, TRPV1 KO mice have decreased latency times when compared to wild-type mice, indicating an antidepressant phenotype (You et al., 2012). Capsazepine is known to enhance antidepressant activity when administered to fluoxetine-treated mice at a sub-threshold dose in the forced swim test (Manna and Umathe, 2012). Desensitization of supraspinal TRPV1 has also been shown to produce an antidepressant-like effect, as evidenced by the reduction in immobility time in the mouse forced swim test following i.c.v. injection of capsaicin at a dose that would have desensitized the receptor (Manna and Umathe, 2012). Further evidence for the involvement of central TRPV1 comes from work demonstrating that intrathecal injections of a TRPV1-desensitising dose of the agonist RTX also reduced immobility in the mouse forced swim test and inhibited the immobility induced by a lower dose of RTX (Abdelhamid et al., 2014). Moreover, the antidepressants, amitriptyline and ketamine, administered intraperitoneally, inhibited the increase in forced swim test immobility (water at 41°C) induced by a low dose of RTX administered subcutaneously (Abdelhamid et al., 2014). These workers also demonstrated that water at 41°C elicited less immobility than cooler water (26 °C), indicating that thermoregulatory sites do not contribute to immobility in the forced swim test. Finally, systemic administration of the TRPV1 agonist olvanil reduced immobility in the rat forced swim test due to desensitisation (Kasckow et al., 2004).

1.7 Overall hypothesis and objectives of the research presented in this thesis

While the evidence reviewed in this introductory chapter (section 1.6) suggests that TRPV1 in the PAG modulates both nociceptive behaviour and anxiety-related behaviour, there is paucity of studies examining its role in hyperalgesia associated with negative affective state such as is exhibited by the WKY rat. Given the well established role of TRPV1 in modulating peripheral and central pain processing, I hypothesised that TRPV1 might be differentially expressed within the columns of the PAG in WKY versus SD rats. Furthermore, pharmacological modulation of TRPV1 in the columns of the PAG might have differential effects on formalin-evoked nociceptive behaviour in stress-hyperresponsive WKY rats, compared with SD rats.

The overall objective of the work presented herein was to improve our understanding of the role of TRPV1 in the interaction between negative affect and pain. The aim of the work presented in the first results chapter (Chapter 2) was to characterise TRPV1 expression in each of the columns of the PAG in the presence or absence of formalin-evoked nociceptive tone in the WKY rat model of hyperalgesia associated with negative affective state, compared with SD controls. Chapters 3-5 examine the role of TRPV1 in each of the different columns of the PAG of WKY versus SD rats in formalin-evoked nociceptive behaviour and associated neurochemical alterations in the RVM and expression of *c-Fos* in the dorsal horn of the spinal cord.

Chapter 2: Characterization of pain- and anxiety-related behaviour and TRPV1 expression in the PAG in the WKY rat model of negative affective state

2.1 Introduction

The adaptive or defensive response mounted by an organism exposed to acute, intense stress leading to decreased pain response is termed stress-induced analgesia (SIA) (Butler and Finn, 2009). Chronic stress, by contrast, generally tends to exacerbate pain in humans and rodents in the phenomenon of stress-induced hyperalgesia (SIH) (Jennings et al., 2014). Chronic stress is also a well-recognised predisposing factor in anxiety disorders and clinical studies have provided evidence that there is high comorbidity between pain and anxiety (Asmundson and Katz, 2009; Atkinson et al., 1991). Furthermore, a persistent anxiety state can exacerbate pain leading to anxiety-induced hyperalgesia (al Absi and Rokke, 1991; Dougher, 1979; Rhudy and Meagher, 2000). Thus, preclinical and clinical studies provide substantial evidence for hyperalgesia associated with chronic stress or anxiety (SIH), but the underlying neuronal mechanisms are still not well understood.

Regarding the role of TRPV1 in SIH, data have demonstrated an increase in TRPV1 mRNA expression and a decrease in CB₁ receptor mRNA expression and AEA levels at the level of the DRG in the WAS model of SIH (Hong et al., 2009b). Another study suggested that subcutaneous injection of corticosterone in situ showed a significant increase in serum corticosterone associated with visceral hyperalgesia by altering the TRPV1/CB₁ receptor expression (Hong et al., 2011). Recently, it has been demonstrated that chronic stress induces visceral and somatosensory hyperalgesia that is associated with differential changes in endovanilloid levels in DRG innervating the pelvic viscera and lower extremities. TRPV1 expression was increased in the DRG of nociceptive C-fibers innervating the pelvic viscera and lower extremities of rats exhibiting hyperalgesia following exposure to chronic, intermittent water avoidance stress (Zheng et al., 2015). Another study also reported that TRPV1 mRNA and protein levels were upregulated in the colon of stressed rats in the water avoidance stress model, suggesting stress-induced visceral hypersensitivity may be mediated via TRPV1 modulation (Van Den Wijngaard et al., 2009). Administration of RTX (Resiniferatoxin; TRPV1 agonist) intraluminally decreased anxiety-like behaviour in an animal model of ulcerative colitis due to desensitisation of TRPV1 (Jinghong Chen, 2015). Abdelhamid et al reported that

TRPV1 agonism transiently decreased musculoskeletal hyperalgesia induced by forced-swim stress exposure (in a water bath of 41⁰C). The musculoskeletal hyperalgesia was completely abolished when the animals were placed in cold water as TRPV1 is a heat-sensitive channel (Abdelhamid et al., 2013). Furthermore, social stress enhances TRPV1 mRNA expression in urinary bladders and systemic administration of capsazepine (TRPV1 antagonist) counteracted such an effect (Mingin et al., 2015).

Recent studies have provided evidence for a role of supraspinal TRPV1 in the modulation of SIH. Intracerebroventricular administration of the TRPV1 antagonist SB 366791 reduced chemical and inflammatory abdominal nocifensive responses (Jurik et al., 2014). TRPV1 mRNA expression is upregulated in the PAG in the rat model of streptozocin-induced diabetic neuropathy (Mohammadi-Farani et al., 2010). TRPV1 mRNA and protein levels were also found to be upregulated in the hippocampus of rats exposed to restraint stress (Navarria et al., 2014). All the above data suggests that TRPV1 does play a role in stress and pain and might represent a novel therapeutic target for the treatment of SIH.

Genetic background is an important moderating influence on the interaction between negative affective state and pain. Herein, I have used the Wistar-Kyoto (WKY) rat model to further our understanding in this area. The WKY inbred rat strain exhibits a stress-hyperresponsive (De La Garza and Mahoney, 2004; A Lahmame et al., 1997), anxiety/depressive-like phenotype (Gentsch et al., 1987; Paré, 1994) and also displays a hyperalgesic response to a variety of noxious stimuli (Burke et al., 2010; Hyland et al., 2015; Moloney et al., 2015; O' Mahony et al., 2013; Rea et al., 2014). Thus, the WKY rat represents a useful model of hyperalgesia associated with negative affective state and may facilitate understanding of the underlying neurobiological mechanisms and identification of novel therapeutic targets for pain and its co-morbidity with affective disorders. To date, there has been a paucity of studies on the role of supraspinal TRPV1 in hyperalgesia associated with negative affective state. Herein, I characterised TRPV1 mRNA and protein expression in the PAG in the WKY rat model of hyperalgesia associated with negative affective state by comparing with stress- and pain-normoresponsive SD rats in the presence and absence of the noxious inflammatory stimulus of formalin. Given the well-established role of the TRPV1 in modulating peripheral and central pain processing, we hypothesised that hyperalgesia in WKY vs. SD rats would be associated with differential expression of TRPV1 within the PAG.

The aims of the experiments described in this chapter were:

- To further characterise behaviour in the WKY rat model of hyperalgesia associated with negative affect, versus SD rats.
- To quantify TRPV1 mRNA expression in the PAG of WKY and SD rats after intra-plantar formalin or saline injection.
- To quantify TRPV1 protein expression in the PAG of WKY and SD rats after intra-plantar formalin or saline injection.

2.2 Materials and Methods:

2.2.1. Animals

For all experiments, male Sprague–Dawley (SD) or Wistar–Kyoto (WKY) rats (260–290 g) (Harlan, UK) were used. Animals were singly housed for the duration of experiments in cages with dimensions 42×26×13cm filled with wood chip bedding (Goldflake Sawdust Bedding Ltd, UK) with access to food (Harlan Teklad global diets chow: ENVIGO RMS, United Kingdom) and water (tap water) ad libitum. Holding rooms were maintained at a constant temperature ($21\pm 2^{\circ}\text{C}$), under standard lighting conditions (12:12-hour light–dark, lights on from 0800 to 2000h) and relative humidity of 40–60%. Experiments were carried out during the light phase between 0800 and 1700h. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway, under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609. All sections of the study adhered to the ARRIVE Guidelines for reporting in animal research (Kilkenny et al., 2010)

2.2.2. Experimental design

Animals were randomly assigned to treatment groups and the sequence of treatments was randomised to control the order of testing. Quantification of TRPV1 gene expression was carried out on tissue generated by previous PhD graduate from the laboratory, Dr. Weredeselam Olango (Olango, 2012). There were 4 experimental groups in that study (Study 1): SD-Saline (n=6), SD-Formalin (n=6), WKY-Saline (n=6), WKY-Formalin (n=6). I then repeated this experiment (Study 2) to characterise TRPV1 protein expression (and included two additional groups of naïve SD and WKY rats). Naïve rats were included in the 2nd study to examine the TRPV1 protein levels in the absence of saline/formalin injection. Thus, 21 male SD rats and 21 male WKY rats were exposed to the open field (Day 7 or 8 post-arrival into the unit), elevated plus maze (EPM) (Day 7 or 8 post-arrival), hot plate (Day 9 post-arrival) and intra-plantar formalin or saline injections (Days 12-15 post-arrival), in that order. Rats in the naïve groups did not receive any intra-plantar injection of formalin or saline and remained in their home cage until sacrifice. This design resulted in 6 experimental groups for the assessment of behaviour and TRPV1 protein expression as follows: SD-Naïve; SD-

Saline (SD-Sal); SD-Formalin (SD-Form); WKY-Naïve; WKY-Saline (WKY-Sal); and WKY-Formalin (WKY-Form) (n=6-7 per group).

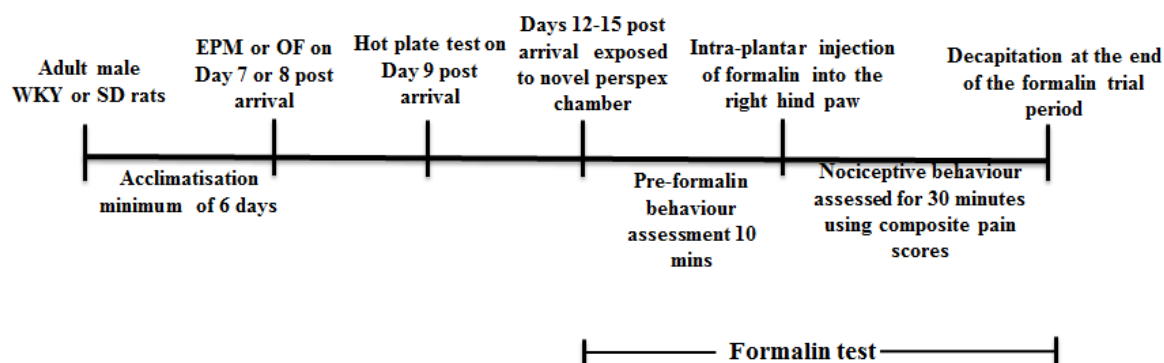


Figure 2.1: Timeline for characterization of pain- and anxiety-related behaviour in SD and WKY rats

2.2.3. Behavioural testing

The behavioural testing and time points were identical for Study 1 and Study 2.

2.2.3.1. Open field test

Behaviour in the open field was assessed once for SD and WKY rats in an alternate manner on the day 7 and 8 post arrival. On the experiment day, each animal was removed from the home cage during the light phase between 0900 h - 1200 h or 1300h to 1600h and placed into a brightly lit (lux 300) novel open field environment (diameter 75 cm and 40cm high walls constructed). 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® XT8.5, Noldus, The Netherlands) for a 5 min period. The open field was cleaned between animals with cleaning solution (Milton:tap water; 1:5). Behaviours assessed included locomotor activity (total distance moved) and duration of time spent (seconds) in the centre zone (45 cm diameter). Reduced time spent in the centre zone is usually interpreted as anxiety-related behaviour.

2.2.3.2. Elevated plus maze (EPM)

The EPM consisted of a plus-shaped wooden maze with two closed arms enclosed by walls (30 cm) and two open arms. Each arm was 50 cm in length and 10 cm in width and the arms were interconnected by a central platform and elevated 50 cm from the

room floor. A video camera was positioned over the maze and the light levels were fixed at 60 lux in the open arms and 25 lux in the closed arms. The EPM test was carried out once for both WKY and SD rats in an alternate fashion (open field was carried out first then 2 min-3 mins later EPM was carried out). On the experiment day (day 7/8 post arrival between 0900 h - 1200 h or 1300h to 1600h), rats were placed on the central platform with their head pointing towards one of the open arms constantly. The rat behaviour was recorded and analysed using a computerized video tracking system (EthoVision® XT7, Noldus, the Netherlands) for a 5 min period. The EPM was cleaned between animals with cleaning solution (Milton: tap water; 1:5). Reduced time spent in the open arms(s) was used as an experimental index of anxiety, whereas the entries into the closed arms are seen as indices of general locomotion. Entries in arms were defined as entry of the rat's centre of gravity into the arms (centre point on the body). Distance moved in each section of the EPM and the percentage distance moved in the OA (Open arms) of EPM was also assessed.

2.2.3.3. Nociceptive responding

2.2.3.3.1 Hot plate test

Hot plate testing was carried out once for SD and WKY rats on day 9 post arrival. Nociceptive responding of SD and WKY rats to an acute thermal stimulus was assessed using this test. The animal was taken from its home cage and placed directly onto a hot plate (IITC Life Science Inc, USA) heated to 54.5 ± 1 °C. The test was carried out on the SD and WKY rats alternately between 1100h and 1400h. Response latency was measured as the time elapsed (s) between placement of the animal on the surface of the hot plate and when the animal first licked either of its hind paws, with a cut-off time of 40s to avoid tissue damage. The hot plate was cleaned between animals with cleaning solution (Milton: tap water; 1:5).

2.2.3.3.2 Formalin test

12-15 days post-arrival of the animals into the unit, the test was carried out. In this test, the rats were placed in a Perspex observation chamber (30×30×40cm; LxWxH, 30lux) for a pre-formalin habituation period of 10 mins during which the effects of novel

environment on general exploratory behaviours were evaluated. After this 10 min pre-formalin trial, rats received an intra-plantar injection of 50 μ L formalin (cat#: F1635, Sigma-Aldrich, Ireland) (2.5% in 0.9% saline) or 0.9% saline into the right hind paw under brief isoflurane anaesthesia (Burke et al., 2010; Rea et al., 2014). Rats were then returned to the same Perspex observation chamber for a period of 30 mins. A video camera located beneath the observation chamber was used to record animal behaviour onto DVD for subsequent analysis. Behaviour was analysed with the aid of Etho-Vision XT8.5 software (Noldus, The Netherlands) by a rater blinded to treatments (i.e. individual scoring the pain behaviour was blinded towards the treatment, strain, saline/formalin injection and other parameters that might influence the behavioural scoring). Formalin-evoked nociceptive behaviour was categorized as time spent raising the formalin-injected paw above the floor without contact with any other surface (C1) and time spent holding, licking, biting, shaking, or flinching the injected paw (C2) to obtain a composite pain score [CPS=(C1+2(C2))/(total duration of analysis period)] (Watson et al., 1997). At the peak of the second phase of the formalin test (30 minutes after formalin injection), rats were killed by decapitation. Pre-and post-formalin hind paw measurement was taken using Vernier callipers, to ensure the inflammation in the paw. Paw inflammation is considered to be a criterion for including the animals for further behavioural analysis. Brains were removed rapidly, snap-frozen on dry ice, and stored at -80°C before microdissection of the columns of the PAG for TRPV1 gene/protein expression.

2.2.4 Tissue harvesting

2.2.4.1. Brain removal

Following decapitation, an incision was made using scissors along the top of the head and the skin pulled back to expose the skull. The optic ridge between the eyes and the back of the skull was broken with rongeurs. Using scissors, a cut was made carefully along the midline of the skull from the back, maintaining pressure away from the brain surface, and the parietal and frontal parts of the skull were removed. The remaining bone along the sinus between the olfactory bulbs and frontal cortex was carefully removed, as was the bone over the nasal cavity and eye socket. The dura mater was removed, the trigeminal nerve was cut and the brain removed from the skull using a curved forceps. The brains were snap-frozen on dry ice and stored at -80°C .

2.2.4.2 Spinal cord removal

After decapitation, an incision was made down the midline of the back of the carcass using a scalpel blade. The skin was removed from the entire dorsal portion of the carcass. The bottom of the rib-cage and the atlanto-occipital joint was identified and an incision was made transversely on both the sides. Then along the length and breadth, the vertebral column is separated from the carcass. Excess tissue surrounding the spinal column was removed for a better grip of the vertebral column. Onto the anterior end (closest to the head) of the spinal column (which bear bigger orifice) a syringe (10ml) without the needle is placed, and the spinal cord is flushed out using freshly prepared phosphate buffered saline at 4⁰C (0.1M PBS) (Kennedy et al., 2013). The L4-L6 region was isolated and cut sagittally and horizontally resulting in an ipsilateral and contralateral dorsal and ventral segment. The dorsal segments were weighed and snap-frozen on dry ice and stored at -80⁰C.

2.2.5 Punch microdissection of columns of PAG tissue

Frozen coronal brain sections (300 μ m in thickness) containing the PAG were cut on a cryostat (MICROM, Germany). A series of 300 μ m-thick sections (from AP -5.80 to -8.72mm relative to bregma) were punched using cylindrical brain punchers (Harvard Apparatus; internal diameter 0.75mm), with the aid of the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1998). PAG columns DLPAG (from AP -5.80 to -8.00mm relative to bregma), VLPAG (from AP -7.30 to -8.30mm relative to bregma) and LPAG (from AP -7.3 to -8.30mm relative to bregma) were punched accordingly. These samples were weighed and stored at -80⁰C before extraction of total RNA for determination of TRPV1 gene expression using quantitative real time – polymerase chain reaction (qRT-PCR) or protein extract preparations for western immunoblotting. The punching and cDNA synthesis for qRT-PCR (Study 1) was done by Dr. Weredeslam Olango and the punching needed for the TRPV1 protein expression (Study 2) was carried out by myself. The range of weights of the punch-dissected tissue per PAG column were: DLPAG: 1.5-2.5mg; VLPAG: 2.7-3.3mg; LPAG: 4.3-5.7mg.

2.2.6 qRT-PCR:

Brain tissues from dlPAG, vlPAG, lPAG, of all the experimental groups (i.e SD-Sal SD-Formalin, WKY-Sal and WKY-Formalin, n=6; Study 1) were analysed by qRT-PCR. Total RNA was extracted from homogenized tissue using a Machery-Nagel extraction kit (Nucleospin RNA II, Technopath, Ireland). This method involved homogenising tissue in 350µl lysis buffer (RA1), containing 1% β-mercaptoethanol (Sigma, Ireland) for 3s using an automated homogenizer (Polytron tissue disrupter, Ultra-Turrax, Germany). Homogenates were kept on ice until transferred to a Nucleospin filter (violet ring), centrifuged at 11000g for 1min and the lysates treated with 350µl of 70% molecular grade ethanol (Sigma, Ireland). Samples were transferred to Nucleospin RNA spin column II (light blue ring) and centrifuged at 11000g for 30s to bind the RNA. After desalting the column membrane with membrane desalting buffer (MDB,), RNA samples were treated with 10µl DNase for 15min at room temperature to remove DNA from the sample. Samples were then serially washed using washing buffers (200µl RA2, 600µl RA3 and 250µl RA3) and RNA was eluted in 20µl of RNAase-free water (Sigma, Ireland). The quantity, purity and quality of RNA were assessed using Nanodrop (ND-1000, Nanodrop, Labtech International, UK). RNA quantity was determined by measuring optical density (OD) at 260nm. RNA quality was determined by measuring the ratio OD260/OD280 where a ratio of approximately 1.8-2.1 was deemed indicative of pure RNA. All mRNA samples showed OD260/OD280 ratios between 1.75 and 2.2 on the Nanodrop. mRNA samples were kept at -800C until required for cDNA synthesis.

RNA samples were equalised to 3,000ng/11µl using RNase-free water. Equal amounts of total RNA (3,000ng in 11µl) from each sample were then reverse transcribed into complementary DNA (cDNA) as follows. Target RNA and primers were combined and denatured by addition of 11µl of RNA and 0.8µl random nanomers (Sigma, Ireland), 0.2µl oligo(dT)15 primers (Medical supply Co, Ireland) and 1µl PCR Nucleotide mix (dNTP mix, Medical supply Co, Ireland) and incubation at 65⁰C for 5 min. The 13 µl of target RNA mix was then mixed with 4µl of 1st strand buffer (Bioscience LTD, Ireland), 1µl of DTT (Bioscience LTD, Ireland), 1µl of recombinant ribonuclease inhibitor (RNaseOUT, Bioscience LTD, Ireland) and 1µl of SuperScript III Reverse Transcriptase (Bioscience LTD, Ireland) to a total reaction volume of 20µl. Two negative controls, reverse transcription reactions were included where reverse

transcriptase or RNA templates were replaced with nuclease-free water. Samples were run on a PCR machine/thermocycler (MJ Research, INC, USA) using steps below:

Annealing: 25 °C for 5min

Extension: 50 °C for 50min

Inactivation: 70 °C for 15min

cDNAs were kept at -20°C until required for quantification by qRT-PCR. TRPV1 gene primers were generated using 3.0 Primer Express software and acquired from Eurofins MWG, UK. The following sequences were used in generating the TRPV1-FAM labelled primers

FORWARD PRIMER: CAGCAGCAGTGAGACCCCTAA

REVERSE PRIMER: TGTCCTGTAGGAGTCGGTTCAA

PROBE: CGTCATGACATGCTTCTCGTGGAACC

VIC-labelled GAPDH (Rn_4308313; Applied Biosystems, UK) was used as the housekeeping gene and endogenous control. Expression of TRPV1 and the endogenous control gene was assessed using an Applied Biosystems ‘StepOne plus’ instrument (Bio-Sciences, Dun Laoghaire, Ireland). A no-template control reaction was included in all assays in order to validate the instrument and the samples in every assay. Samples were run as duplicates and in a multiplex assay. Reactions were performed for each sample and C_t values were normalized to the housekeeping GAPDH gene expression. The relative expression of TRPV1 to GAPDH was calculated by using the $2^{\Delta\Delta C_t}$ method. The $2^{\Delta\Delta C_t}$ values for each sample were then expressed as a percentage of the mean of the $2^{\Delta\Delta C_t}$ values for the control group (SD-SAL).

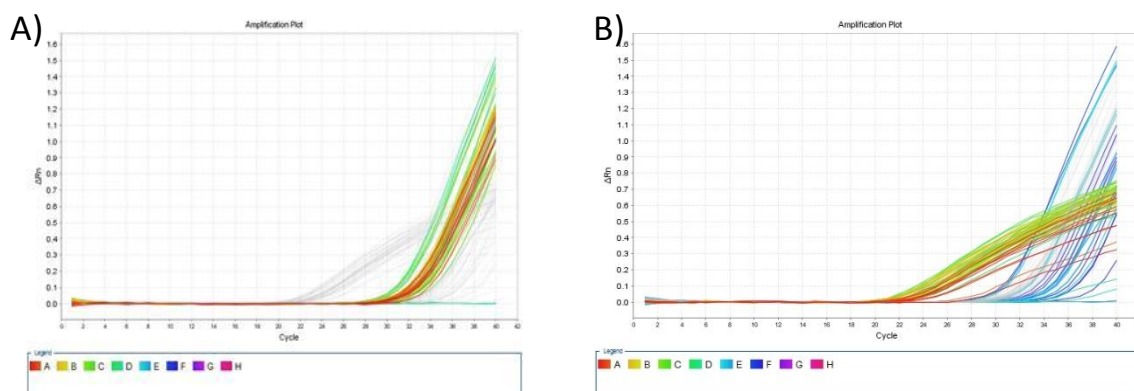


Figure 2.2: Amplification plots in the StepOne plus for (A) TRPV1 (B) GAPDH gene expression

2.2.7 Western immunoblotting:

Brain tissues from DLPAG, VLPAG, LPAG, of all the experimental groups (i.e SD-Naïve, SD-Saline, SD-Formalin, WKY-Naïve, WKY-Saline and WKY-Formalin, n=6; Study 2) were analysed by western immunoblotting. Frozen brain tissue was 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 Na₃VO₄, 10mmol/L NaF containing 1% protease inhibitor cocktail [Sigma-Aldrich, Ireland]) in a 1.5mL microcentrifuge tube (100µl-LPAG 75µl-DLPAG and VLPAG). After homogenisation the microcentrifuge tube was placed on the shaker for 45 mins at 4⁰C for the RIPA lysis buffer to lose the proteins bound either to plasma membrane/ nuclear membrane and then centrifuged at 13,200 rpm (14,000g) (Eppendorf Centrifuge 5415R Stevenage, UK) for 20min at 4⁰C 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, 2mg/ml) were prepared in water. The Bradford assay consisted of adding 250ul 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 1.5mg/ml after determining the protein concentration. 8µl of sample loading buffer is added to 24µl of protein sample (36µg 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 are vortexed quickly and then boiled at 95⁰C 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 35 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. 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 mouse polyclonal antibody to the TRPV1 receptor (cytoplasmic N-terminus) (1:200 Cat# 75-254 (purified form) Antibodies Inc, USA). The antibody has been validated in the initial trial of western blotting and also immunofluorescence- Refer to section 2.2.7) and mouse monoclonal antibody to β -actin (1:10000 Cat# 5441; Sigma-Aldrich, Ireland) diluted in 5% milk/0.05% TBST overnight at 4⁰C. Post incubation period, the membrane was washed in washing buffer (0.1% TBST) for 3 x 10 min washes. After the washing, the membranes are transferred onto a plain surface and based on the molecular weight ladder the blot was trimmed closer to 70kDa mark, this step is essential as both the antibodies are raised in mouse, and trimming of the blot prevents competitive binding of the secondary antibody to the high-abundancy β -actin band (~42-kDa) which would likely impact negatively on detection of the less abundant TRPV1 band (~100-kDa). Membranes were then incubated in secondary antibody solution containing IR-Dye goat anti-mouse (k700) (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 TRPV1 receptor protein expression (~100-kDa) and β -actin (~42-kDa) 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 TRPV1 (Refer to image 2.7 D), due to phosphorylation of the receptor as has been reported previously (Navarria et al., 2014; Tóth et al., 2005). In addition, personal communication from F.Ionatti, postdoctoral researcher from Prof. Vincenzo Di Marzo's laboratory has confirmed the presence of multiple bands in the desired region using the antibody from Antibodies Inc (Cat# 75-254). For each sample, the average intensity of the two bands for TRPV1 was calculated and then the ratio of TRPV1 receptor intensity to β -actin intensity was then calculated for that sample. The intensity of each sample is expressed as a percentage of the average of the SD-naïve group. Full details of the composition of all buffers/solutions used are provided in Appendix 1.1.

2.2.8 Statistical analysis

The SPSS statistical package (IBM SPSS v20.0 for Windows; SPSS, Inc., Chicago, IL) was used to analyse all data. Shapiro–Wilk test confirmed that all data with the

exception of defecation data were normally distributed. Levene's test was used to determine homogeneity of variance or if data did not pass Levene's test then parametric statistics were still employed provided that (a) the dependent variable was continuous and (b) largest variance is no more than 3 times the smallest (Dean and Voss, 1999). Student's unpaired, two-tailed t-test was used to compare the hot plate, open field and EPM. Formalin test data and expression data (mRNA and protein expression) were analysed using two-factor analysis of variance (ANOVA), with the factors of strain and formalin. Posthoc pairwise comparisons were made with Fisher's LSD when appropriate (i.e. when $P < 0.05$ in ANOVAs). Defecation (pellet number) data were nonparametric and were analysed using Kruskal-Wallis test followed by pairwise group comparisons with Mann-Whitney U tests. Data were considered significant when $P < 0.05$. Results are expressed as group mean \pm standard error of the mean (SEM) for parametric data and median (with interquartile range) for nonparametric data. For the purposes of presentation and readability in this and all subsequent results chapters, only the P values for significant ANOVA results are provided in the Results section text, along with those for the posthoc pairwise group comparisons. All F, DF and P values for both significant and non-significant ANOVAs, t-tests and Kruskal-Wallis tests, are provided in the figure/table legends in each chapter.

2.3 Results:

2.3.1 WKY rats exhibited reduced locomotor activity and higher anxiety-related behaviour, compared with SD rats

In the open-field test, WKY rats exhibited significantly lower locomotor activity (distance moved), compared with SD rats (Fig 2.3A WKY vs SD, $***P < 0.001$), but there was no significant difference between WKY and SD rats for time spent in the inner zone (Fig 2.3B). In the EPM test, WKY rats spent significantly less time in the open arms (Fig 2.3C SD vs WKY $***P < 0.001$) and exhibited reduced distance moved (Fig 2.3D vs SD, $***P < 0.001$), compared with SD rats.

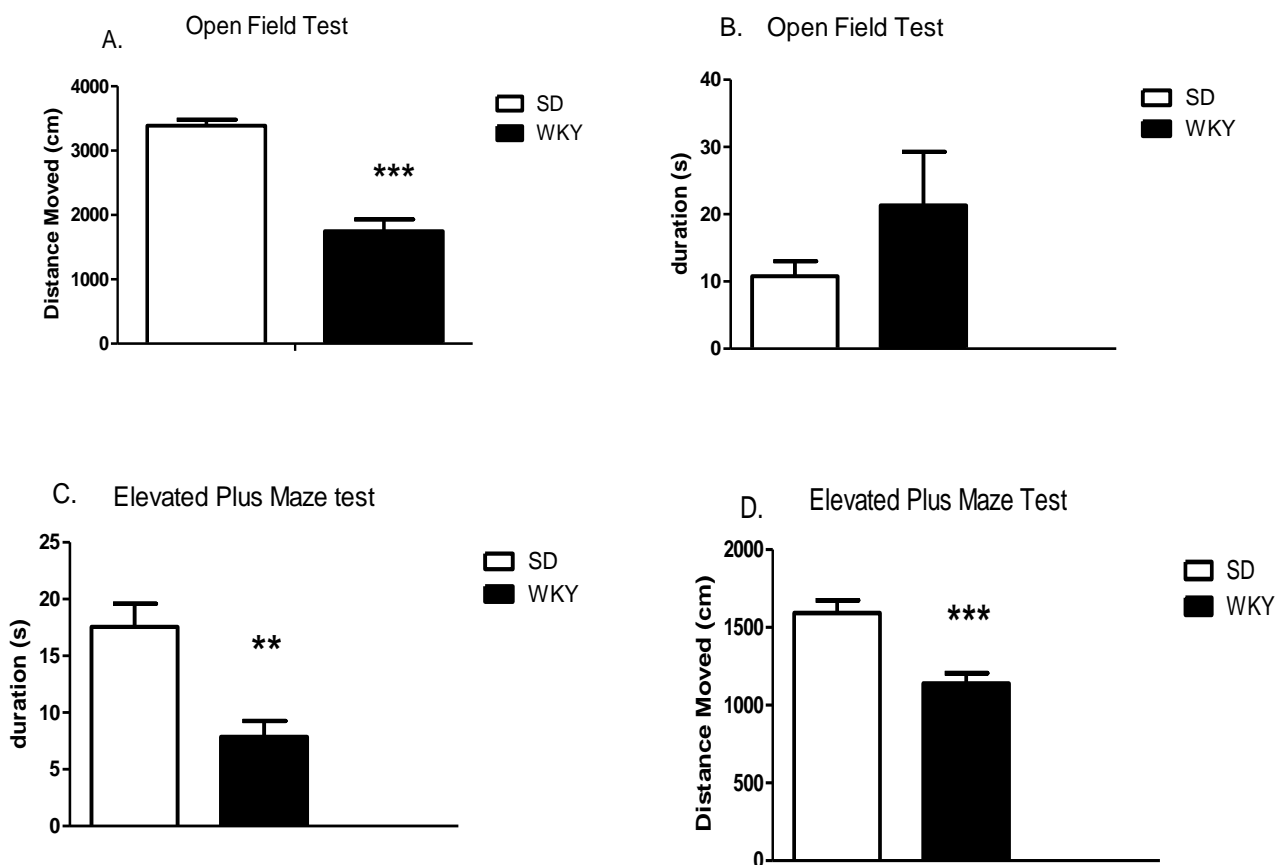


Figure 2.3: Comparison of anxiety-related behaviour in WKY and SD rats in OF and EPM. (A) distance moved in open field: ($t_{26} = 7.711$, $***P < 0.001$) (B) time spent in centre zone of open field: ($t_{26} = 1.363$, $P = 0.185$) (C) time spent in open arms on the EPM: ($t_{26} = 3.915$, $**P < 0.01$) (D) total distance moved in the EPM: ($t_{26} = 4.313$, $***P < 0.001$). Data are expressed as mean \pm SEM ($n = 21$ rats per group).

2.3.2 WKY rats exhibited lower general exploratory and locomotor behaviours when compared to SD rats in a novel environment (preformalin behavioural assessment)

During the 10 min habituation period in the novel Perspex arena prior to intra-plantar formalin/saline administration, WKY rats displayed significantly lower rearing and grooming activity when compared to SD rats (Table 2.1 WKY vs SD, $P < 0.05$). The distance moved by WKY rats was lower than SD rats, but this effect did not reach statistical significance. The number of faecal pellets excreted by WKY rats was significantly higher than SD rats (Table 2.1 WKY vs SD, $P < 0.05$).

Group	Distance moved (cm)	Grooming (s)	Rearing (s)	Defecation (Pellet number)
SD	1470±82.7	20±4	67±9	0
WKY	1381.8±41.8	9±2*	44±6*	0 (0-1)*

Table 2.1: Comparison of general exploratory and locomotor behaviour in WKY and SD rats in novel perspex arena prior to formalin injection. Distance moved: Student's unpaired, two-tailed t-test ($t_{26} = 0.956$, $P = 0.348$), grooming: Student's unpaired, two-tailed t-test ($t_{26} = 2.388$, $*P < 0.05$), rearing: Student's unpaired, two-tailed t-test ($t_{26} = 2.193$, $*P < 0.05$), defecation: Mann-Whitney U test ($U = 2.409$, $*P < 0.05$). Data are expressed as mean ± SEM for parametric data and median (interquartile range) for nonparametric data (n = 14 rats per group).

2.3.3 WKY rats exhibited increased nociceptive responses to noxious thermal and inflammatory stimuli when compared to SD rats

WKY rats showed reduced latency to lick/withdraw either of their hind paws on the hot plate test, compared with the SD rats (Fig 2.4A WKY vs SD, $P < 0.05$). Two-way ANOVA revealed significant strain effect ($P < 0.01$) and formalin effect ($P < 0.001$) in the formalin test. Formalin-treated WKY and SD rats exhibited higher composite pain scores over the 30 min trial (Fig 2.4B, SD-Saline vs SD Formalin, $P < 0.001$; WKY-Saline vs WKY-Formalin, $P < 0.001$) when compared to their respective saline-treated

counterparts. Furthermore, WKY rats exhibited higher formalin-evoked nociceptive behaviour when compared to SD counterparts (Fig 2.4B SD-Formalin vs WKY-Formalin, $P < 0.001$).

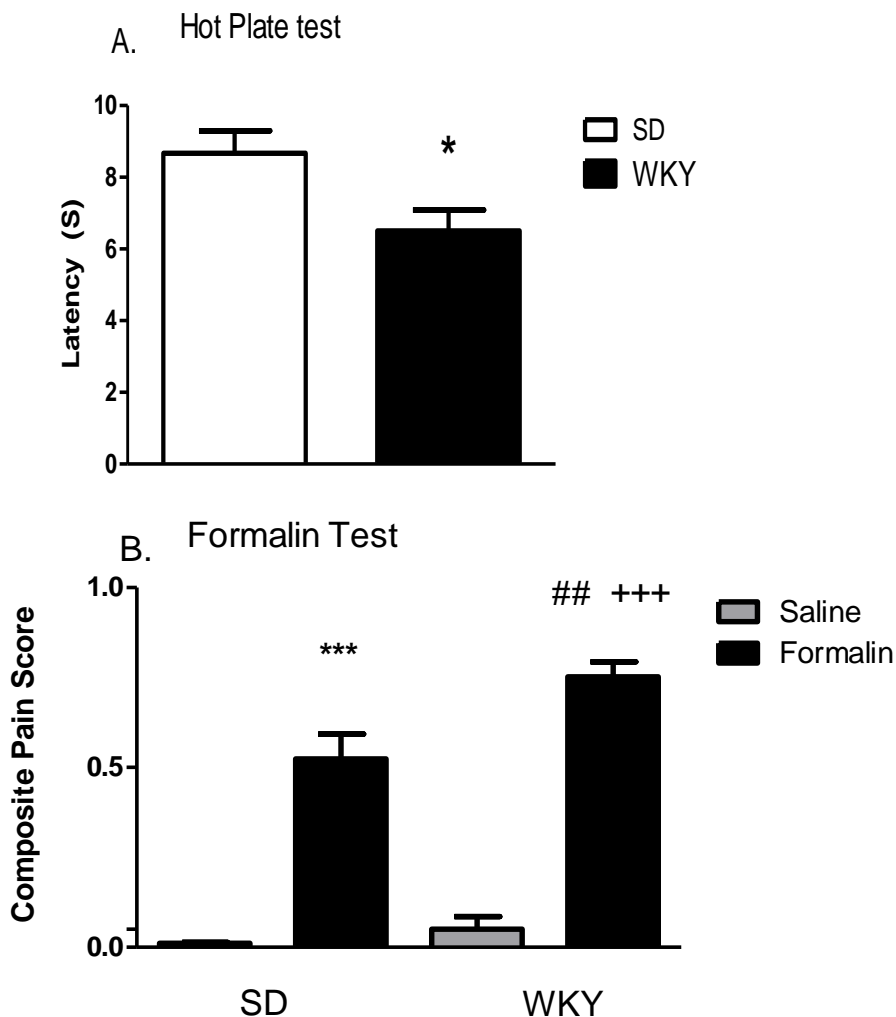


Figure 2.4: Hyperalgesic behaviour of WKY rats to (A) noxious thermal stimulus on hot plate test: ($t_{26} = 2.534$, $*P < 0.05$). Data are mean \pm SEM (n = 21 rats per group). (B) noxious inflammatory stimulus of intra-plantar formalin for a time period of 30 min: Two-way ANOVA: strain ($F_{1,27} = 9.93$, $P < 0.01$); formalin ($F_{1,27} = 190.96$, $P < 0.001$) and formalin x strain interaction ($F_{1,27} = 4.61$, $P = 0.046$), followed by Fisher's LSD post hoc test ($***P < 0.001$ vs. SD-Sal, $##P < 0.01$ vs. SD-Form & $+++P < 0.001$ vs. WKY-Sal). Data are expressed as mean \pm SEM (n=6-7).

2.3.4 Comparison of general locomotor activity, paw-diameter difference and defecation between WKY and SD rats post-formalin injection

Two-way ANOVA revealed significant strain effect ($P < 0.05$), formalin effect ($P < 0.01$) and strain X formalin interaction ($P < 0.001$) on the distance moved during the post-

formalin trial. Formalin-treated SD rats exhibited higher locomotor activity during the formalin test when compared to their saline-treated counterparts (Table 2.2 SD-Formalin vs SD-Saline $P < 0.001$). Formalin-treated WKY rats exhibited lower locomotor activity than their SD counterparts (Table 2.2 WKY-Formalin vs SD-Formalin $P < 0.01$). Two-way ANOVA revealed significant formalin effect ($P < 0.01$) ($P < 0.05$) on the grooming and rearing respectively during the post-formalin trial. General exploratory behaviours such as rearing and grooming decreased (Table 2.2 WKY-Formalin vs WKY-Saline $P < 0.05$) upon formalin administration in WKY rats when compared to saline treatment. There were no such effects of formalin in SD rats (Table 2.2). Rats that received intra-plantar formalin injection had significantly higher paw diameter difference irrespective of the strain when compared to their respective saline injected counterparts (Fig 2.5 $P < 0.001$ SD-Saline vs SD-formalin and WKY – Saline vs WKY-formalin).

Group	Distance moved (cm)	Grooming (s)	Rearing (s)	Defecation (Pellet number)
SD-Saline	1035.5±187.5	38±12	27.6±14	1 (0-1)
SD-Formalin	3015.8±305.0 ^{***}	13±5	17±9	1 (0-2)
WKY –Saline	1513.2±278.2	47±16	31±7.5	0 (0-1)
WKY-Formalin	1335.2±43.4 ^{##}	10±4 ⁺	2±1 ⁺	1 (0-1)

Table 2.2: General exploratory behaviours in WKY rats when compared to SD rats during the post-formalin trial. Distance moved: Two-way ANOVA effects of strain ($F_{1,24}=6.975$, $P < 0.05$); formalin ($F_{1,24}=15.66$, $P < 0.01$) and strain X formalin interaction ($F_{1,24}= 22.458$, $P < 0.001$); Grooming: Two-way ANOVA effects of strain ($F_{1,24}=0.103$, $P=0.752$); formalin ($F_{1,24}=8.742$, $P < 0.01$) and strain X formalin interaction ($F_{1,24}=0.345$, $P=0.563$); Rearing: Two-way ANOVA (effects of strain $F_{1,24}=0.411$, $P=0.528$); formalin ($F_{1,24}=4.586$, $P < 0.05$) and strain X formalin interaction ($F_{1,24}=1.039$, $P=0.318$); followed by Fisher's LSD post hoc test (^{***} $P < 0.001$ vs SD-Saline, ^{##} $P < 0.01$ vs SD-Formalin, ⁺ $P < 0.05$ vs WKY-Saline); Defecation: Kruskal-Wallis analysis of variance by rank ($X^2 = 2.491$, $P = 0.477$). Data are expressed as mean \pm SEM for parametric data and median (interquartile range) for nonparametric data (n =6-7 rats per group).

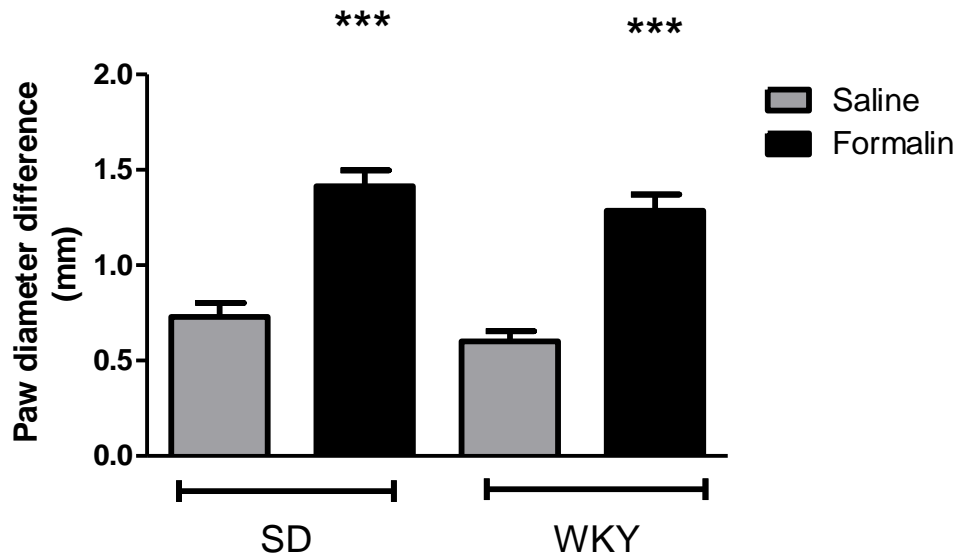


Figure 2.5: Difference in paw diameter (mm) pre- and post-formalin injection. Two-way ANOVA (effects of strain ($F_{1,24} = 2.919$, $P = 0.18$); treatment ($F_{3,24} = 89.7$, $P < 0.001$) and strain X treatment interaction ($F_{1,24} = 0.001$, $P = 0.99$) followed by Fisher's LSD post hoc test. *** $P < 0.001$ vs SD counterpart. Data are expressed as mean \pm S.E.M, $n=6-7$. (SD: Sprague-Dawley, WKY: Wistar-Kyoto).

2.3.5 TRPV1 gene expression in columns of the PAG in SD versus WKY rats

Two-way ANOVA revealed significant strain X formalin interaction ($P < 0.05$) in DLPAG strain effect ($P < 0.01$) in the VLPAG and formalin effect ($P < 0.01$) in the LPAG for TRPV1 mRNA expression. TRPV1 mRNA levels were significantly lower in the DLPAG (Fig 2.6B SD-Saline vs. WKY-Saline, $P < 0.05$), and higher in the LPAG (Fig 2.6D SD-Saline vs. WKY-Saline, $P < 0.05$) of saline-treated WKY rats, compared with SD counterparts. There were no significant differences in TRPV1 mRNA expression in the VLPAG between the two strains (Fig 2.6C). Intra-plantar injection of formalin significantly reduced TRPV1 mRNA levels in the DLPAG of SD rats, but not WKY rats (Fig 2.6B SD-Saline vs. SD-Formalin, $P < 0.05$). In contrast, formalin injection significantly increased TRPV1 mRNA levels in the VLPAG of SD rats, but not in WKY rats (Fig 2.6C SD-Saline vs. SD-Formalin, $P < 0.001$) and had no significant effect on levels of TRPV1 in the LPAG of either SD or WKY rats (Fig 2.6D).

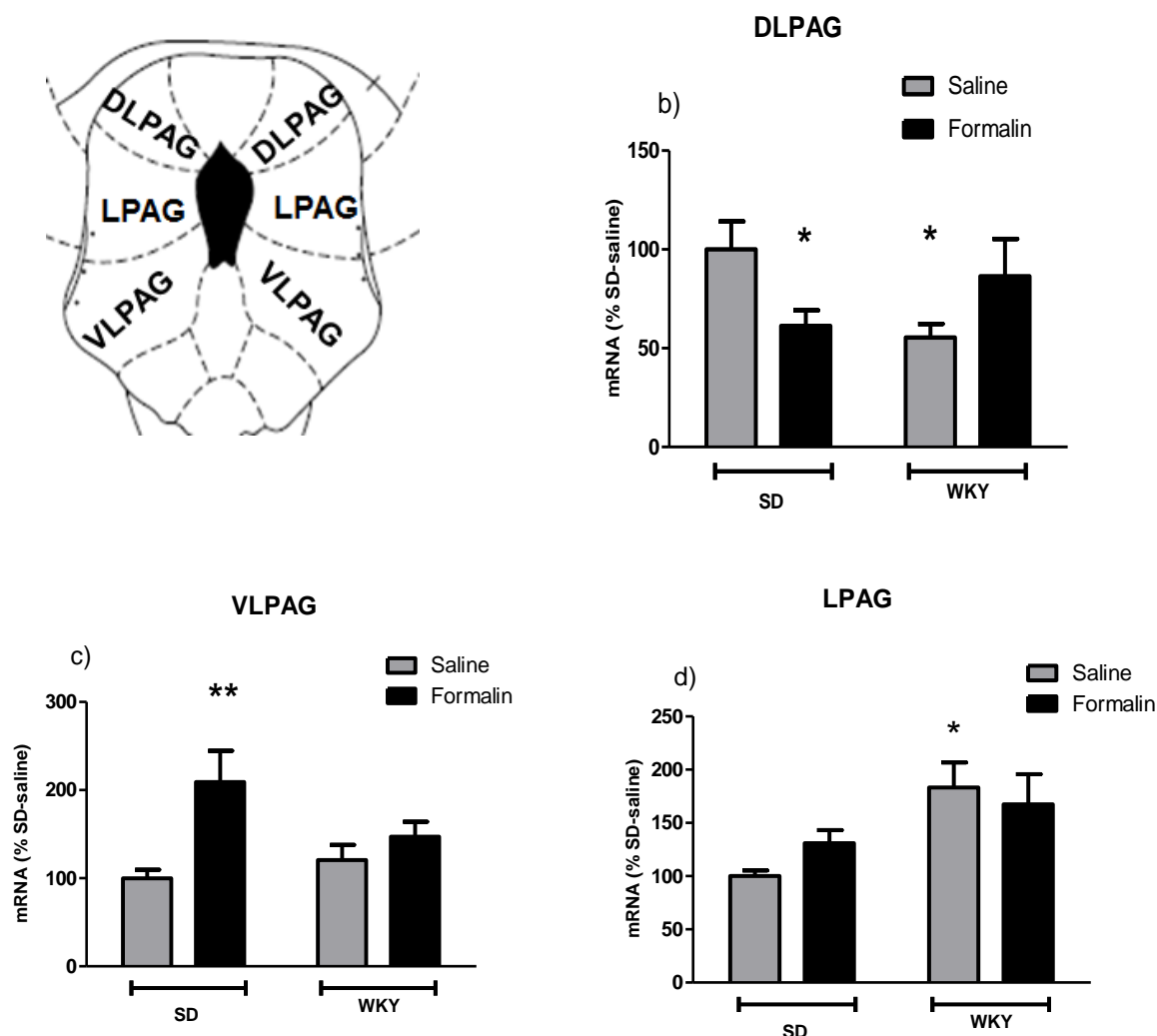


Figure 2.6: (a) Schematic representation of columns in the PAG (b) TRPV1 mRNA levels in the DLPAG of SD and WKY rats that received intra-plantar injection of either saline or formalin. Two-way ANOVA effects of strain: $F_{1,18} = 0.646$, $P = 0.432$; formalin: $F_{1,18} = 0.100$, $P = 0.756$ and strain \times formalin interaction: $F_{1,18} = 8.209$, $P < 0.05$) followed by Fisher's LSD post hoc test ($*P < 0.05$, vs SD-SAL). (c) TRPV1 mRNA levels in the VLPAG of SD and WKY rats that received intra-plantar injection of either saline or formalin. Two-way ANOVA effects of strain: $F_{1,18} = 8.714$, $P < 0.01$; formalin: $F_{1,18} = 0.137$, $P = 0.715$ and strain \times formalin interaction: $F_{1,18} = 1.346$, $P = 0.261$) followed by Fisher's LSD post hoc test ($**P < 0.01$ vs SD-Saline). (d) TRPV1 mRNA levels in LPAG of SD and WKY rats that received intra-plantar injection of either saline or formalin. Two-way ANOVA effects of strain: $F_{1,18} = 0.840$, $P = 0.371$; formalin: $F_{1,18} = 9.10$, $P < 0.01$ and strain \times formalin interaction: $F_{1,18} = 3.382$, $P = 0.082$) followed by Fisher's LSD post hoc test ($^{\$}P < 0.05$ vs SD-Saline). Data are expressed as mean \pm SEM (n = 5 - 6 rats per group).

2.3.6 TRPV1 protein expression in columns of the PAG in SD versus WKY rats

There were no significant changes in TRPV1 protein expression upon saline/formalin administration either in the DLPAG (Fig 2.7A) or in LPAG (Fig 2.7C). There was a significant strain effect in the two-way ANOVA for the VLPAG, but pairwise post hoc comparisons did not reach statistical significance. There were no significant effects of strain on TRPV1 protein expression in the DLPAG or LPAG.

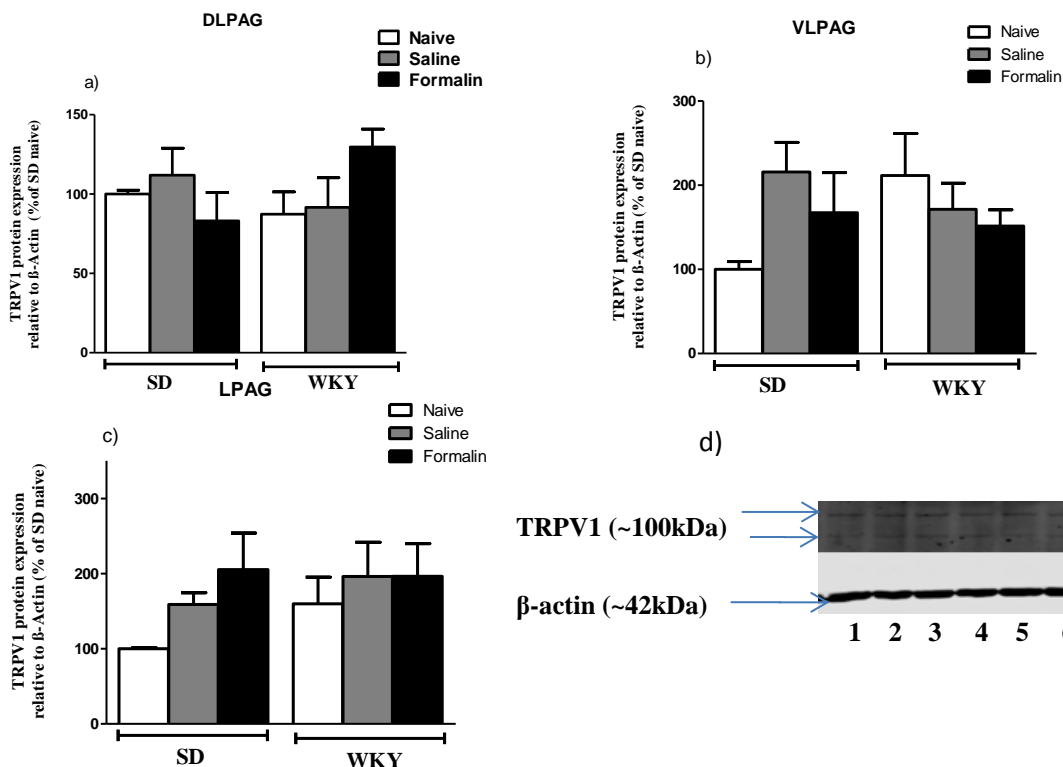


Figure 2.7: TRPV1 protein expression in columns of the PAG in SD versus WKY rats (a) TRPV1 protein levels in the DLPAG of SD and WKY rats that received an intra-plantar injection of either saline or formalin or no injection (naïve). Two-way ANOVA effects of strain: ($F_{1,35} = 0.134, P = 0.717$; treatment: $F_{2,35} = 1.114, P = 0.340$ and strain \times treatment interaction: $F_{2,35} = 0.484, P = 0.621$) (b) TRPV1 protein levels in the VLPAG of SD and WKY rats that received an intra-plantar injection of either saline or formalin or no injection (naïve). Two-way ANOVA effects of strain: $F_{1,35} = 8.714, P < 0.01$; treatment: $F_{2,35} = 0.137, P = 0.715$ and strain \times formalin interaction: $F_{2,35} = 1.346, P = 0.261$). (c) TRPV1 protein levels in LPAG of SD and WKY rats that received an intra-plantar injection of either saline or formalin or no injection (naïve). Two-way ANOVA effects of strain: $F_{1,34} = 1.941, P = 0.173$; treatment: $F_{2,34} = 2.743, P = 0.079$ and strain \times treatment interaction: $F_{2,34} = 0.034, P = 0.966$). (d) Representative western immunoblot image, showing the bands for TRPV1 receptor and β -actin for six groups 1.SD-Naïve, 2. SD-Saline, 3.SD-Formalin, 4.WKY-Naïve, 5.WKY-Saline, 6.WKY-Formalin. Data are expressed as mean \pm SEM (n = 5 - 7 rats per group).

2.4 Discussion

In the present study, changes in TRPV1 expression in the PAG of WKY versus SD rats that express differential anxiety-, depression- and pain-related behaviour were investigated. WKY rats exhibited anxiety-like behaviour in the open field test and elevated plus maze test. Moreover, when first exposed to the novel environment of the Perspex formalin test arena, the WKY rats exhibited lower rearing and grooming and higher defecation when compared to SD rats, all considered hallmarks of anxiety-related behaviour. In terms of nociceptive responding, WKY rats exhibited lower response latencies to respond in the hot-plate test and greater formalin-evoked nociceptive behaviour, when compared to SD rats, confirming the hyperalgesic phenotype. The qRT-PCR data indicated that TRPV1 mRNA expression was significantly lower in the DLPAG and higher in the LPAG of WKY rats compared with SD counterparts. There were no significant differences in TRPV1 mRNA expression in the VLPAG between the two strains. Intra-plantar injection of formalin significantly decreased TRPV1 mRNA levels in the DLPAG and increased TRPV1 mRNA levels in the VLPAG of SD, but not WKY rats. However, there were no significant changes in TRPV1 protein expression in any of the 3 columns of PAG with respect to strain and/or formalin treatment.

The duration of time spent in the inner zone of the open field, which is a measure of anxiety-related behaviour, was not significantly different between SD and WKY rats. This result contrasts with previous studies in our laboratory (Burke et al., 2010; Olango, 2012) and others (Braw et al., 2006; Malkesman et al., 2005; McAuley et al., 2009; Smith et al., 2016), which demonstrated that WKY rats spend significantly less time in the inner zone compared with SD rats. However, in the present study, the WKY rats spent most of the time freezing in the inner zone of the open field when compared to the SD rats. This behaviour can be attributed to hyperresponsive (i.e. freezing) nature of WKY rats when exposed to a novel environment such as the open field (Burke et al., 2010; Olango, 2012). This is reflected in the data for total distance moved throughout the entire open field which was significantly lower in WKY rats compared with SD counterparts, in agreement with previous studies from our laboratory (Burke et al., 2010; Olango, 2012) and others (Braw et al., 2006; Malkesman et al., 2005; McAuley et al., 2009; Smith et al., 2016). WKY rats exhibited anxiety-like behaviour in the EPM test i.e. WKY rats spent less time in the open arms and distance moved was less when compared to SD rats. The data were in line with the results of previous experiments

from our laboratory (Burke et al., 2010; Olango, 2012) and also from others (Gentsch et al., 1987; A Lahmame et al., 1997). The Open arm entries of SD and WKY rats were similar and this might be due to the fact that the open field test was conducted first (for half of the cohort) and is known to be a stressor when compared to EPM. During the 10 min pre-formalin trials in the Perspex formalin test arena, the rearing and grooming of WKY rats were lower when compared to their SD counterparts, further suggesting that the WKY rats exhibited increased anxiety-like behaviour in this novel environment and supporting previous findings (Burke et al., 2010; Paré, 1994). Furthermore, the faecal output of the WKY rats during the pre-formalin trial was higher than that of SD rats, another index of greater anxiogenic behaviour in WKY rats when exposed to a novel environment (Hyland et al., 2015). WKY rats exhibited lower thermal nociceptive threshold when compared to SD rats as reported previously (Burke et al., 2010; Olango, 2012; Schaap et al., 2012). WKY rats displayed increased formalin-evoked nociceptive behaviour compared to SD rats, confirming the inflammatory hyperalgesic behaviour in this inbred rat strain as demonstrated previously by studies from our laboratory (Burke et al., 2010; Rea et al., 2014). The distance moved in the arena was increased in SD and WKY rats after intraplantar formalin administration suggesting that general locomotor activity is influenced by a noxious inflammatory stimulus. The fact that WKY rats exhibited lower locomotor activity but yet higher formalin-evoked nociceptive behaviour compared with SD rats suggests that their hyperalgesic behaviour is expressed independently of, and despite, their hypo locomotor activity and likely reflects exacerbated nociception in this inbred strain. Taken together, the results presented herein confirm that WKY rats display greater anxiety- and pain-related behaviour in line with the previous studies from our lab and others (Braw et al., 2006; Burke et al., 2010; Gentsch et al., 1987; Hyland et al., 2015; A Lahmame et al., 1997; McAuley et al., 2009; Rea et al., 2014; Smith et al., 2016).

In SD rats, formalin-evoked nociceptive behaviour was associated with reduced TRPV1 mRNA expression in the DLPAG. WKY rats also had lower levels of TRPV1 mRNA expression in the DLPAG compared with SD rats and it is possible that this may explain their propensity to respond in a hyperalgesic manner to formalin injection. Moreover, we observed a formalin-induced increase in TRPV1 mRNA expression in the DLPAG of WKY rats and hypothesise that this may represent a compensatory change in an attempt to reduce pain behaviour in the WKY strain. These hypotheses were subsequently tested in the pharmacological studies described in later chapters of this

thesis. In the LPAG, although saline-injected WKY rats had higher levels of TRPV1 mRNA expression compared with SD counterparts, formalin injection had no effect on TRPV1 mRNA levels in either strain. In comparison, formalin-evoked nociceptive behaviour in SD rats was associated with higher TRPV1 mRNA expression in the VLPAG. Formalin-treated WKY rats had lower TRPV1 mRNA expression in the VLPAG compared with SD formalin treated rats. Such an effect on TRPV1 mRNA expression in WKY rats may constitute a compensatory change to counter the hyperalgesic response to formalin exhibited by this strain. Taken together, the data also provide evidence for differences in the expression of TRPV1 mRNA in specific PAG columns, between WKY and SD rats, suggesting that TRPV1 expression and/or functionality in the PAG plays a role in hyper-responsivity to inflammatory pain in a genetic background prone to negative affect. These data provide evidence for rapid, dynamic formalin-evoked alterations in TRPV1 gene expression in pain-related brain regions of SD and WKY rats.

There were no significant differences in PAG TRPV1 protein expression between SD and WKY rats and no significant effects of formalin injection within or between the two strains, although some non-significant trends were observed and it is possible that these may have some biological significance. The results were in contrast to the mRNA expression in the SD and WKY rats. Discrepancies between the mRNA expression and protein expression may be due to various factors; first, the TRPV1 mRNA differences reported might not be translated into differences at the functional protein level at the 30 min time point where we harvested the tissue; second, TRPV1 protein expression was difficult to determine because of the multiple bands expressed around the same region of the blot as the TRPV1 band of interest, which in turn might be due to phosphorylation, dimer formation (transient receptor potential ankyrin 1 or actanomin1 receptors), or glycosylation of TRPV1 (Mandadi et al., 2006; Tóth et al., 2005; Veldhuis et al., 2012). Finding an appropriate antibody against TRPV1 to use in the western blotting study was a challenge. A number of papers have questioned the presence of TRPV1 in the brain (Fogaça et al., 2012; Madasu et al., 2015; Martins et al., 2014). However, recently, Navarria et al., demonstrated that TRPV1 is present in the hippocampus, and is upregulated in Wistar rats exposed to restraint stress (Navarria et al., 2014). We used the same antibody used by Navarria et al., after working unsuccessfully with different bodies from Santa Cruz and Chemicon.

In conclusion, WKY rats exhibited greater anxiety-like behaviour and hyperalgesia to

noxious thermal and inflammatory stimuli when compared to SD rats, in line with previous experiments from our laboratory and others. The nociceptive behavioural changes were associated with differential TRPV1 mRNA expression in specific columns of the PAG in both SD and WKY rats. TRPV1 mRNA expression was higher in LPAG and lower in DLPAG of WKY rats when compared to SD counterparts, PAG column-specific and strain-specific expression of this receptor. An acute, noxious inflammatory stimulus produced rapid changes in TRPV1 mRNA expression but not significant alterations in protein expression. These data provide evidence for rapid, dynamic formalin-evoked alterations in TRPV1 gene expression within the PAG columns of SD and WKY rats. In the next chapters, I tried to establish whether these alterations underlie differential formalin-evoked nociceptive behaviour in the two rat strains.

Chapter 3: The effects of pharmacological modulation of TRPV1 in the dorsolateral periaqueductal grey on formalin-evoked nociceptive behaviour in Wistar-Kyoto versus Sprague-Dawley rats

3.1 Introduction

The localisation of TRPV1 in the DLPAG has been confirmed by immunohistochemistry (Casarotto et al., 2012a; Cristino et al., 2006; McGaraughty et al., 2003), qRT-PCR (refer to chapter 2), western blotting (refer to Chapter 2), gene reporter approach (Cavanaugh et al., 2011), pharmacological studies (Batista PA, Fogaça MV, 2015; Casarotto et al., 2012b; Fogaça et al., 2012; Mascarenhas et al., 2015, 2013; McGaraughty et al., 2003; Moreira et al., 2008; Palazzo et al., 2002; Terzian et al., 2009). Pharmacological studies have confirmed that TRPV1 in the DLPAG is involved in pain processing (Madasu et al., 2015; McGaraughty et al., 2003; Palazzo et al., 2002) and anxiety-related behaviour (Casarotto et al., 2012a; Fogaça et al., 2012; Madasu et al., 2015; Terzian et al., 2009). However, there is a paucity of data on the role of TRPV1 in pain associated with negative affect. The aim of the work described in this chapter was to study the role of TRPV1 in the DLPAG in hyperalgesia associated with negative affect, and associated alterations in *c-Fos* expression, neurotransmitter levels and endocannabinoid levels in key regions regulating pain, namely the RVM and dorsal horn of spinal cord.

The DLPAG does not receive spinal afferent input directly so does not play a direct role in ascending pain transmission (Keay et al., 1997). The DLPAG has no somatic or visceral inputs arising from the spinal cord or nucleus of the solitary tract (NTS) (Keay and Bandler, 2001; Holstege and Kuypers, 1982; Holstege, 1991). Although, the DLPAG has no direct medullary projections to the RVM, it indirectly influences the RVM via projections to the cuneiform nucleus which in turn projects to the RVM (Holstege and Kuypers, 1982; Holstege, 1991; Keay and Bandler, 2001; Mitchell et al., 1988; Mitchell et al., 1988; Redgrave et al., 1987; Redgrave et al., 1988), and can thus modulate descending pain transmission. The DLPAG has dense projections to hypothalamic nuclei mediating defensive responses (Cameron et al., 1995). In turn, neurons from the ventromedial hypothalamus and dorsal preamillary nucleus also project to the DLPAG (Canteras, 2002; Canteras and Swanson, 1992). Retrograde tracing studies showed that the DLPAG projects onto the nucleus reuniens of the thalamus (Risold et al., 1997). DLPAG stimulation with nitric oxide (NO) donors leads

to activation of anterior cingulate pathway suggesting a role within an ascending pathway with the nucleus reuniens as a relay station (Risold et al., 1997; Semenenko and Lumb, 1992). The DLPAG also receives projections from the dorsal raphe nucleus at the level of oculomotor nucleus (Vertes, 1991). The projections from the dorsal raphe nucleus to the DLPAG are serotonergic and known to modulate the activity of neurons involved in defensive behaviour (Lovick, 1994). The DLPAG receives direct neuronal input from the CeA (Rizvi et al., 1991; Vianna et al., 2003). Stimulation of the BLA, which projects to the CeA, produced sympathetic responses which were similar to responses produced by direct DLPAG activation (Soltis et al., 1998). Furthermore, intra-DLPAG glutamate injection increased *c-Fos* protein expression in the BLA (Ferreira-Netto et al., 2005), suggesting bidirectional connectivity between the amygdala and DLPAG. These functional neuroanatomical studies suggest that the DLPAG is an important component of the descending pain pathway. Indeed, capsaicin, when injected into the DLPAG, increased the latency of nociceptive responding to noxious heat, indicating that stimulation of TRPV1 within this area of the descending inhibitory pain pathway can cause antinociception (Palazzo et al., 2002). Intra-DLPAG microinjection of capsaicin was followed by a decrease in the tail flick-related ON cell burst activity in the RVM and an increase in tail flick latency. In another study, due to desensitization of the receptor (due to prolonged exposure to capsaicin), antinociceptive effects of intra-DLPAG capsaicin correlating with increased OFF cell activity in the RVM was reported (McGaraughty et al., 2003). These studies support a role of TRPV1 in the DLPAG in nociception.

TRPV1 is expressed in a key population of inhibitory GABAergic interneurons of the substantia gelatinosa. In these interneurons, TRPV1 activation decreases the expression of AMPA receptors, therefore reducing neuronal activity. This leads to a reduction in the inhibition of spinothalamic tract neurons of the deep lamina, which is likely to tonically increase the transmission of nociceptive signals from the spinal cord to higher brain centers (Kim et al., 2012), exacerbating pain perception. TRPV1 sensitization is facilitated by descending RVM serotonergic inputs, through the activation of the 5-HT₃ receptor (Kim et al., 2014). Starowicz et al have already shown that activation of TRPV1-expressing glutamatergic neurons of the VLPAG inhibits nociceptive transmission by enhancing pain inhibition and suppressing pain facilitation mediated by OFF and ON cells, respectively. Additionally, a role for tonic regulation of pain was attributed to TRPV1 in the VLPAG-RVM circuit as the administration of I-RTX, a

specific and potent TRPV1 antagonist, induces hyperalgesia and decreases both glutamate release and OFF cell activity in the RVM. Thus, in the present studies, the neurotransmitters GABA, glutamate and serotonin were studied in the key components of the descending pain transmission such as the RVM and dorsal horn of spinal cord to evaluate the pharmacological modulation of TRPV1 on RVM and dorsal horn of the spinal cord.

Chemical or electrical stimulation of the DPAG induces panic or escape-like responses (De Oliveira et al., 2001; Finn et al., 2003; Krieger and Graeff, 1985; Lim et al., 2011; Vianna et al., 2001). TRPV1 in the DLPAG also modulates anxiety-related and panic-like behaviour in rodents. Administration of capsaicin and capsazepine directly into the DLPAG produced anxiolytic effects in the EPM due to desensitisation and blockade, respectively, of TRPV1 in the DLPAG of rats (Terzian et al., 2009). In another study, intra-DLPAG administration of capsaicin increased anxiety-related behaviour in mice in the EPM due to activation of TRPV1 (Mascarenhas et al., 2013). Blockade of TRPV1 in the DLPAG with the antagonists SB366791 or capsazepine decreases panic-like responses in rats (Almeida-Santos et al., 2013; Casarotto et al., 2012a; Lisboa and Guimarães, 2012b). Capsaicin-induced activation of TRPV1 potentiates glutamatergic transmission in the DLPAG (Xing and Li, 2007). Intra-DLPAG administration of a NMDA receptor agonist-induced defensive responses by facilitating glutamatergic transmission via TRPV1 (Bertoglio et al., 2006). Furthermore, in PAG slices, TRPV1 facilitates excitatory neurotransmission upon activation by AEA. Conversely, inhibitory neurotransmission is facilitated by AEA activation of CB₁ receptors (Kawahara et al., 2011). Fogça et al. have shown that the lack of an anxiolytic effect of higher AEA doses is due to facilitation of glutamate release in the DLPAG, probably via activation of TRPV1 (Fogça et al., 2013). Evidence suggests that TRPV1 and CB₁ receptors play opposite roles in the modulation of anxiety-, and pain-related behaviour (Casarotto et al., 2012a; Maione et al., 2007). Activation of CB₁ receptors in the DLPAG reduced fear expression in contextual fear conditioning (Resstel et al., 2008) and activation of TRPV1 had an opposite effect (Uliana et al., 2016). Olango et al reported that there were no differences in CB₁ receptor mRNA expression or the levels of AEA or N-acyl ethanolamines (OEA and PEA) in the DLPAG of WKY rats when compared to SD rats, 30 minutes following intra-plantar injection of saline or formalin (Olango, 2012). In contrast, analysis of TRPV1 receptor expression within the PAG presented in Chapter 2 of this thesis demonstrated differential expression of TRPV1 mRNA in the DLPAG of

WKY and SD rats in the presence and absence of formalin-evoked nociceptive tone. TRPV1 mRNA expression was lower in the DLPAG of saline-treated WKY rats when compared to SD counterparts. Formalin administration significantly decreased TRPV1 mRNA expression in the DLPAG of SD rats, but not in WKY rats. Thus, I hypothesized that altered expression and/or functionality of TRPV1 at the level of the DLPAG might be involved in hyperalgesia associated with a negative affective state in the WKY rat.

The specific aims of the studies described in this chapter were:

- To investigate the effects of pharmacological modulation of TRPV1 in the DLPAG on formalin-evoked nociceptive behaviour in WKY rats when compared to SD rats.
- To investigate whether pharmacological modulation of TRPV1 in the DLPAG alters *c-Fos* mRNA expression in the dorsal horn of spinal cord of formalin-injected WKY and SD rats.
- To assess alterations in levels of neurotransmitters and endocannabinoids in the RVM and dorsal horn of the spinal cord of formalin-injected WKY and SD rats following pharmacological modulation of TRPV1 in the DLPAG.

3.2 Materials and Methods

3.2.1. Animals

For all experiments, male SD and/or WKY rats (260-290g) (Harlan, UK) were used. Animals were group housed before surgery and singly housed post-surgery. Holding rooms were maintained at a constant temperature ($21\pm 2^{\circ}\text{C}$), under standard lighting conditions (12:12-hour light–dark, lights on from 0800 to 2000h) and relative humidity of 40–60%. Experiments were carried out during the light phase between 0800 and 1700h. Food and water were available *ad libitum*. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway, under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609. All sections of the study adhered to the ARRIVE Guidelines for reporting in animal research (Kilkenny et al., 2010)

3.2.2 Experimental design

In this chapter, I investigated the effects of pharmacological modulation of TRPV1 in the DLPAG on formalin-evoked nociceptive behaviour in WKY and SD rats. Male SD and WKY rats ($n=5-7$) were implanted bilaterally under isoflurane anaesthesia with stainless steel guide cannulae targeting the DLPAG. On the test day, animals received bilateral intra-DLPAG injections of either vehicle (100% DMSO), the TRPV1 agonist capsaicin (CAP; 6nmoles/0.2 μL), the TRPV1 antagonist 5'-IRTX (0.5nmoles/0.2 μL) or co-administration of capsaicin and 5'-IRTX, and were placed in the formalin test arena for 10 minutes before intra-plantar formalin injection (2.5%, 50 μl) under brief isoflurane anaesthesia. All the treatment groups had equal n numbers before cannula verification. Rats were then returned to the formalin test arena and behaviour was recorded for a period of 60 minutes. In this and all other experiments described in this thesis, animals were randomly assigned to drug treatment groups and the sequence of drug treatments was randomized to control for the order of testing. Rats were killed by decapitation immediately following behavioural testing. A 0.3 μL volume of 1% fast green dye was microinjected via the guide cannulae, and brains and spinal cords were rapidly removed, snap-frozen on dry ice, and stored at -80°C until injection site verification.

	Drug treatment			
	Vehicle	Capsaicin	5'-IRTX	Capsaicin+5'-IRTX
SD (n)	6	5	5	5
WKY (n)	7	7	6	5

Table 3.1: n numbers per group that received intra-DLPAG injections bilaterally post cannula verification.

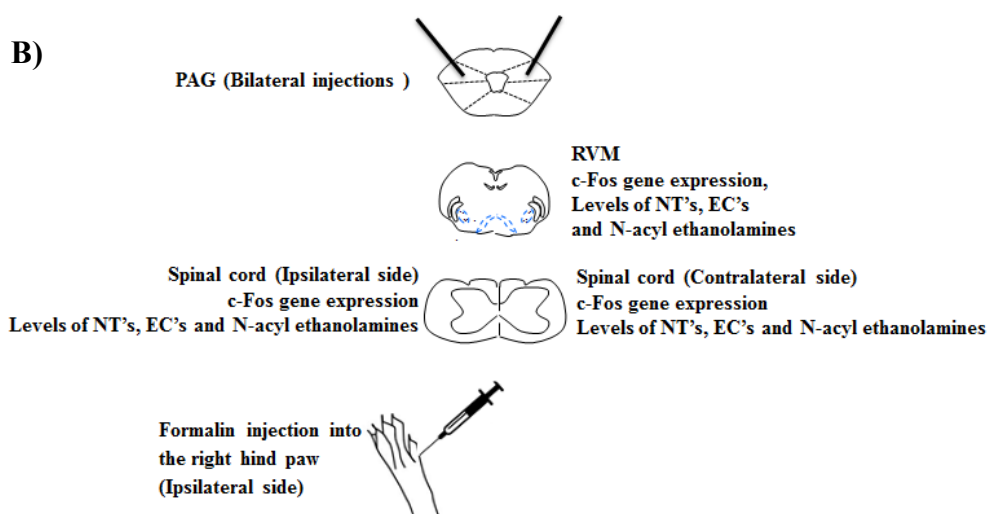
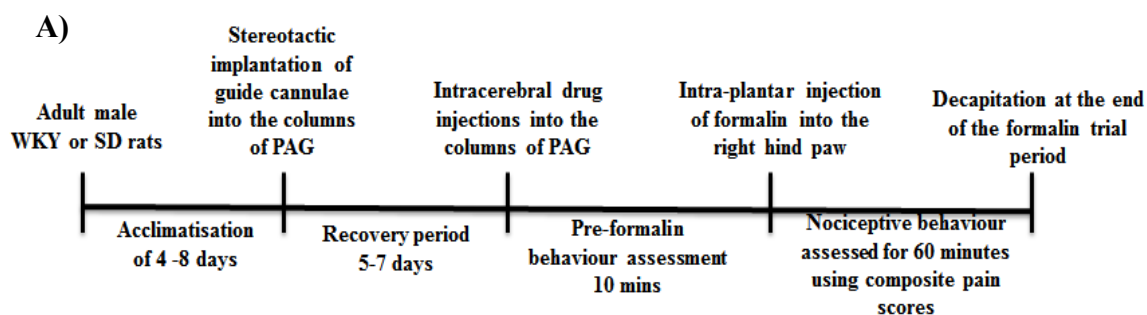


Figure 3.1: A) Timeline for experiments in this and subsequent chapters involving intracerebral drug injections into the columns of PAG B) Diagrammatic representation of methodology carried out

3.2.3 Cannulae implantation:

After acclimatization for 4-8 days after delivery, the rats were placed under brief isoflurane anaesthesia (2-3% in O₂, 0.5L/min), stainless steel guide cannulae (9mm length, Plastics One Inc., USA) were stereotactically implanted bilaterally 1mm above the DLPAG. The following coordinates were used for implanting cannulae in SD rats: AP = ((difference from Bregma to lambda) X 0.91mm) from Bregma, ML = ±1.9mm at an angle of 10°, DV = 4.8mm from the meningeal dura matter; WKY: AP = ((difference from Bregma to lambda) X 0.91mm) from Bregma, ML = ± 1.8mm at an angle of 10°, DV = 5.0mm from the meningeal dura matter according to the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998). The 9mm cannulae were permanently fixed to the skull using stainless steel screws and carboxylate cement (Durelon TM, USA). A stylet made from stainless steel tubing (9mm, 31 G) (Plastics One Inc., USA) was inserted into the guide cannulae to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (5ml/kg, s.c., Rimadyl, Pfizer, UK), was administered before the surgery to manage postoperative analgesia. To prevent postoperative infection, rats received a single daily dose of the antimicrobial agent enrofloxacin (2.5ml/kg, s.c., Baytril, Bayer plc, UK) on the day of surgery and a subsequent 3 days. Following cannulae implantation, the rats were housed singly and allowed at least 5 days recovery prior to experimentation. During this recovery period, the rats were handled, cannulae checked, and their body weight and general health monitored once daily.

3.2.4 Drug preparation

The TRPV1 agonist capsaicin (CAP) was purchased from TOCRIS (UK). The TRPV1 antagonist 5-Iodo-Resiniferatoxin (5'-IRTX) was bought from Abcam (UK). Formalin and DMSO were purchased from Sigma-Aldrich (Ireland). For intra-PAG microinjections, CAP and 5'-IRTX were prepared to concentrations of 6nmol and 0.5nmol per 0.2 µL respectively in DMSO vehicle (dimethylsulfoxide, 100%). For co-administration of CAP and 5'-IRTX we prepared 2X concentrations of CAP and 5'-IRTX in DMSO and then combined them to give final concentrations equal to those of the drugs administered alone. The doses of capsaicin and 5'-IRTX were chosen based on previous studies demonstrating their efficacy following direct injection into the columns of the PAG (Maione et al., 2007, 2006; McGaraughty et al., 2003; Starowicz et al., 2007).

3.2.5 Microinjections

Drugs were microinjected manually into the DLPAG in a volume of 0.2µL using an injector and Hamilton syringe attached to 50-cm-long polyethylene tubing (0.75mm outside diameter, 0.28mm inside diameter, Harvard Apparatus, UK) to minimise handling and enable injections to be carried out while the rats remained in the home cage. Drugs were microinjected over a period of 1min and the needle was left in position for a further 1min to allow diffusion of the drug before the cannula was withdrawn.

3.2.6. Formalin test

On the day of the experiment, rats were placed in a Perspex observation chamber (30×30×40cm; LxWxH, 30lux) covered with black cardboard on the sides and without a lid. After intracerebral injection of drug or vehicle into the right and left DLPAG and the effects of drug treatment on general exploratory behaviours were evaluated for 10 mins. After this 10 min pre-formalin trial, rats received an intra-plantar injection of 50 µL formalin (2.5% in 0.9% saline) into the right hind paw under brief isoflurane anaesthesia (2-3% in O₂, 0.5L/min). Rats were then returned to the same Perspex observation chamber for a period of 60 mins. Refer to Chapter 2 Formalin test (2.2.3.3.2 Formalin test) for detailed description

3.2.7 Tissue harvesting

3.2.7.1. Brain removal

Refer to section 2.2.4.1 Brain removal in Chapter 2

3.2.7.2 Spinal cord removal

Refer to section 2.2.4.2 Brain removal in Chapter 2

3.2.8. Histological verification of microinjection sites

The sites of intra-cerebral microinjection were determined before data analysis and only those rats that had cannulae correctly positioned in both the right and left DLPAG were included in the final analysis. Brain sections with fast-green dye mark were collected on a cryostat (30µm thickness), mounted on gelatinised glass slides, and counterstained with cresyl violet to locate the precise position of microinjection sites under light microscopy. Cryosections containing the right DLPAG mounted on glass slides briefly

dipped in distilled water followed by 5min in 0.1% Cresyl Violet (Sigma-Aldrich, Ireland) and then were dehydrated in graded alcohols as follows: 1 min in 50% ethanol, 1 min 70% ethanol, 2 min in 100% ethanol, 2 min in Xylene, and then 5 min in xylene. Drops of DPX mountant for microscopy (VWR International Ltd., England) were then put onto the slides after which the slides and stained sections were covered with a glass coverslip. The precise position of the injector tips were confirmed under a light microscope.

3.2.9. Tissue grinding

Dissected spinal cords were removed from the -80°C freezer, remained frozen, and were ground in pestle and mortar on dry ice, into a fine powder and split into 2 aliquots (~10mg of ground tissue per aliquot) for assay of neurotransmitters and endocannabinoids by liquid chromatography with tandem mass spectrometry (LC-MS/MS) or *c-Fos* mRNA analysis by qRT-PCR.

3.2.10. Quantitative real-time PCR

qRT-PCR was carried out as described in detail in Chapter 2 (Section 2.2.6). Single-stranded cDNA products were then analysed by real-time quantitative PCR using the Applied Biosystems Step One plus Real-Time PCR System (Applied Biosystems, UK). Taqman gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probes were used (Lifesciences, Ireland). Assay IDs for the genes examined were as follows for rat *c-Fos* (4331182-Rn00487426_g1) and VIC-labelled GAPDH (Rn_4308313; Applied Biosystems, UK) was used as the housekeeping gene and endogenous control. A no-template control reaction was included in all assays in order to validate the instrument and the samples in every assay. Samples were run as duplicates and in a multiplex assay. Reactions were performed for each sample and C_t values were normalized to the housekeeping GAPDH gene expression. The relative expression of target genes to GAPDH was calculated by using the $2^{\Delta\Delta C_t}$ method. The $2^{\Delta\Delta C_t}$ values for each sample were then expressed as a percentage of the mean of the $2^{\Delta\Delta C_t}$ values for the control group (SD-Vehicle-contralateral side).

3.2.11. Quantitation of neurotransmitters, endocannabinoids and N-acylethanolamines in the spinal cord tissue assessed using LC-MS/MS:

The concentrations of the endocannabinoids, AEA and 2-AG, as well as the related N-acylethanolamines N-palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA), and neurotransmitters glutamate, GABA and serotonin were measured simultaneously in the same assay run in RVM and spinal cord tissue by LC-MS/MS. Non-deuterated and deuterated endocannabinoids were procured from Cayman Chemicals (Biosciences, UK) ((Non-deuterated CAY62160- 2-AG, CAY90050-AEA, CAY90350-PEA, CAY90265-OEA) (deuterated CAY362160-2-AG(d8), CAY390050-AEA(d8), CAY10007824-PEA(d4), CAY10007823-OEA(d2))). Non-deuterated and deuterated neurotransmitters were procured from a variety of suppliers. Non-deuterated neurotransmitters were from procured from Sigma Chemicals (Ireland) A2129- GABA, G1251-glutamate, H9523-serotonin. Deuterated neurotransmitters for GABA and glutamate were procured from CDN isotopes (Canada) D1828- GABA (D6), D2193-glutamate (D5). The deuterated serotonin was procured from Alsachim (France) M760-serotonin (D4). Tissue was first homogenized in 200µL of 100% acetonitrile (Fisher, Ireland) containing known fixed amounts of deuterated internal standards of endocannabinoids (2.5ng AEA-d8, 50ng 2-AG-d8, 2.5ng PEA-d4 and 2.5ng OEA-d2) and 10 µL of the deuterated neurotransmitter solution (5µg GABA-d6, 5µg Glutamate-d5 and 1ng Serotonin-d4). The volume was made up to 260 µL using 100% acetonitrile (equal to the volume of standards). The samples were sonicated for 3-4 seconds on ice using ultrasonic homogeniser/sonicator (Mason, Ireland). Homogenates were centrifuged at 14,000g for 15 min at 4°C (Hettich® centrifuge Mikro 22R, Germany) and the supernatant was collected. 40 µL of the supernatant was transferred into a plastic capped HPLC vial (VWR, Ireland). A 10 point standard curve was constructed. To construct the curve, 40µl of 100% ACN were added to tube #10 (the tube with the highest concentration of standards) and 50µl were added to all other tubes. Positive displacement pipettes were used at all times. The standard curve was constructed using serial 1/2 dilution by adding 50µl of endocannabinoids (25ng for PEA, OEA and AEA + 250ng for 2-AG) and 10µl of D0 mixture of neurotransmitters (100µg of glutamate and GABA, 10ng of serotonin) to tube #10, vortexing, then taking out 50µl and transferring to the next tube (#9) containing 50µl acetonitrile. Following vortexing, 50 µl were removed and pipetted into the next tube (#8) etc. The process was repeated until tube #1 using the same pipette tip. 50µl was discarded from tube #1. Thus, all 10 tubes had 50µl of a mixture of endocannabinoids and neurotransmitters. All standard curve

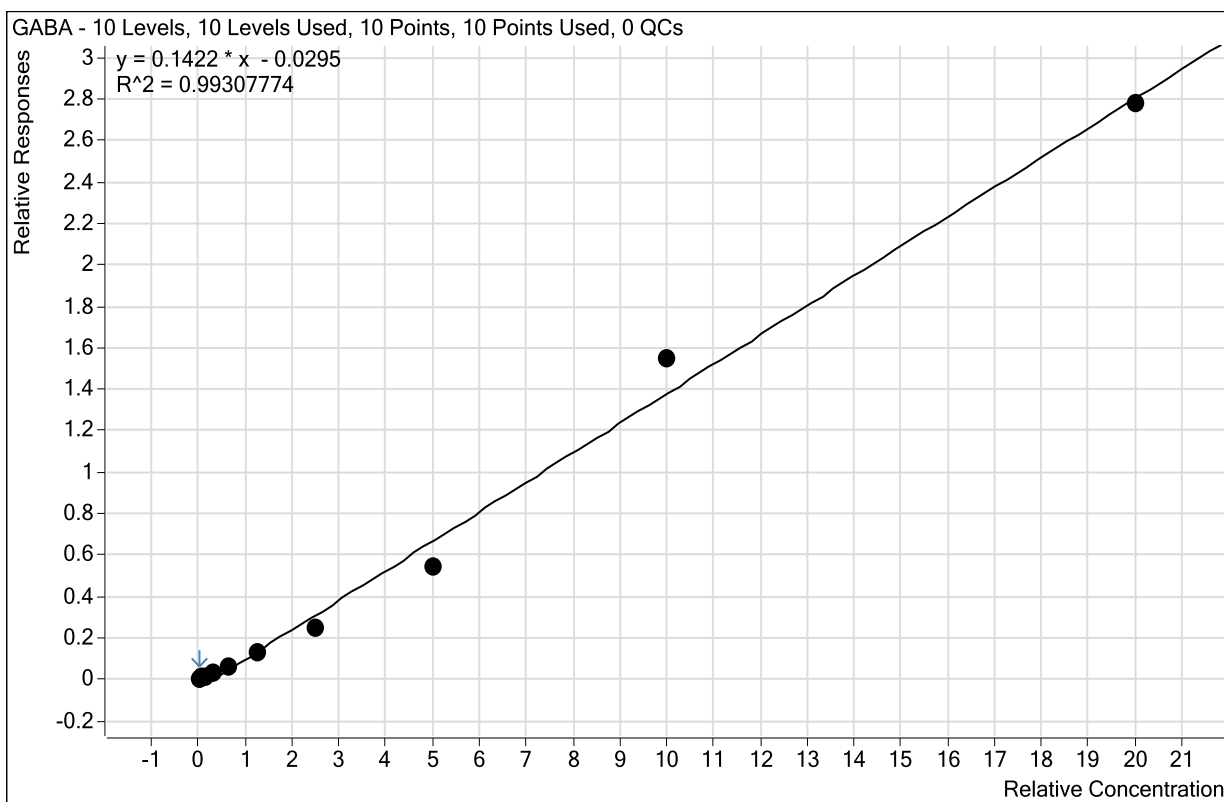
tubes were spiked with 200µl of deuterated endocannabinoid mixture (2.5ng deuterated PEA, OEA and AEA and 50ng deuterated 2-AG as internal standards) and 10 µl of deuterated neurotransmitter mixture (5µg of glutamate and GABA, and 1ng of serotonin) (Refer to Figure 3.2 for standard curves). 6µL per sample/standard was injected onto an ATLANTIS T3-3µ column (2.1 X 100 mm internal diameter) (Waters, UK) from a cooled autosampler maintained at 4°C (Agilent Technologies Ltd., Ireland). A double blank (100% acetonitrile) was also included in between each and every standard during the run to minimise the risk of analyte carryover from standard to standard at the upper range of the curve and six double blanks were included after the highest concentration point on the curve to avoid carryover onto the samples. A quality control sample prepared from the whole rat brain homogenate was included with each run to allow for monitoring of inter- runs variability and instrument performance over time. The quality control was run after all the samples. Mobile phases consisted of A (high-performance liquid chromatography [HPLC]–grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 200µl/minute. Reverse-phase gradient elution began initially at 100% A and at 3.1 min was ramped up to 65% B (and 35% A), it was constant over for a min (until 4.1 min) and then ramped up to 100% B over 8 min and remained constant until 17 mins (Refer to table 3.2). After 17 min, re-calibration is carried out where 100% of B is ramped up until next 10 mins. The first 6 mins of the HPLC gradient is optimised for the neurotransmitters panel of MRM transitions and then at 6.1 minutes the instrument starts looking out for endocannabinoids MRM transitions. Analyte detection was carried out in electrospray positive ionization mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, Ireland) for all the neurotransmitters and endocannabinoids. 10 minute reequilibration time was allowed between each standard or sample injection. Retention times of targets and internal standards were almost identical, but the deuterated internal standard eluted slightly before the corresponding target so the peaks were manually integrated for each and every sample (Refer to Figure 3.3). Quantitation of each analyte was performed using MassHunter Quantitative Analysis Software (Agilent Technologies, Ireland). The limit of quantification was 1.32pmol/g, 12.1pmol/g, 1.5pmol/g, 1.4pmol/g for AEA, 2-AG, PEA and OEA, respectively. The limit of quantification was 0.45nmol/g and 0.3nmol/g, 0.3nmol/g, 0.25nmol/g for GABA, glutamate and serotonin, respectively. 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 performed by 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 with 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.

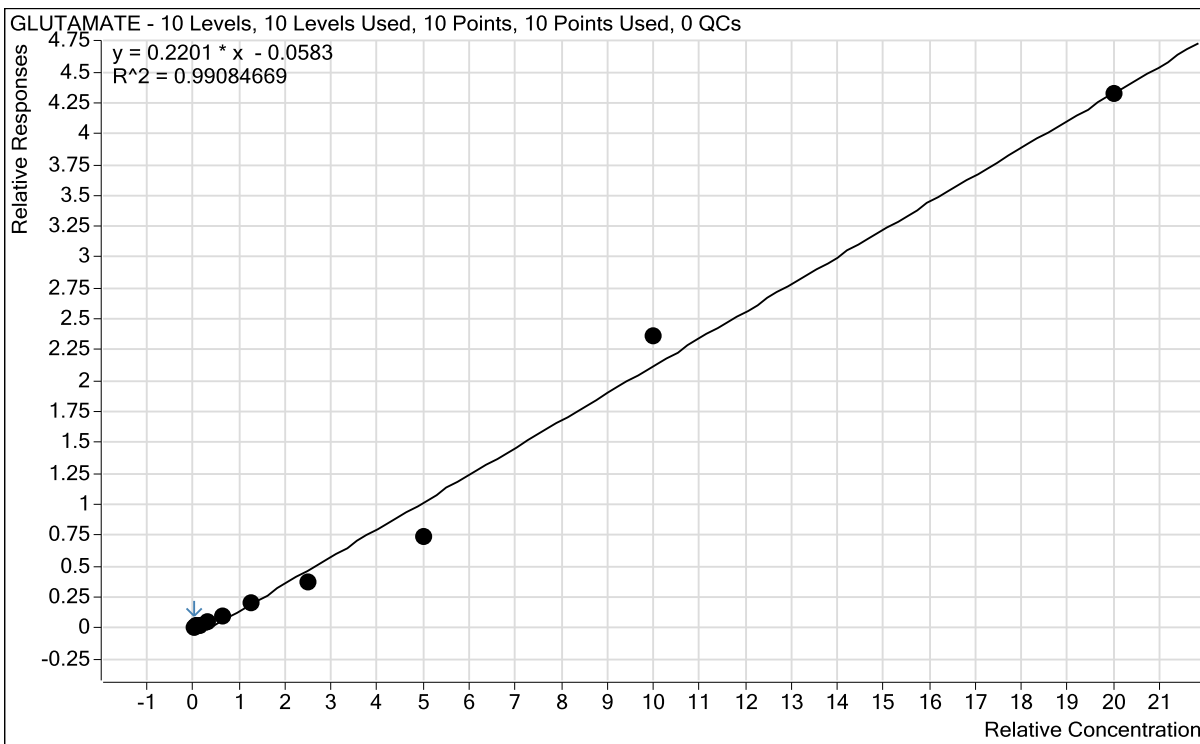
Time	B%	Flow
0.00	0.0	200.000
3.00	0.0	200.000
3.10	65.0	200.000
4.10	65.0	200.000
8.00	100.0	200.000
17.00	100.0	200.000

Table 3.2: HPLC gradient conditions. Time (in minutes), flow (at a rate of 200 $\mu\text{l}/\text{min}$) and B % (Solvent B% ramped up overtime) at a max pressure of 400 bar.

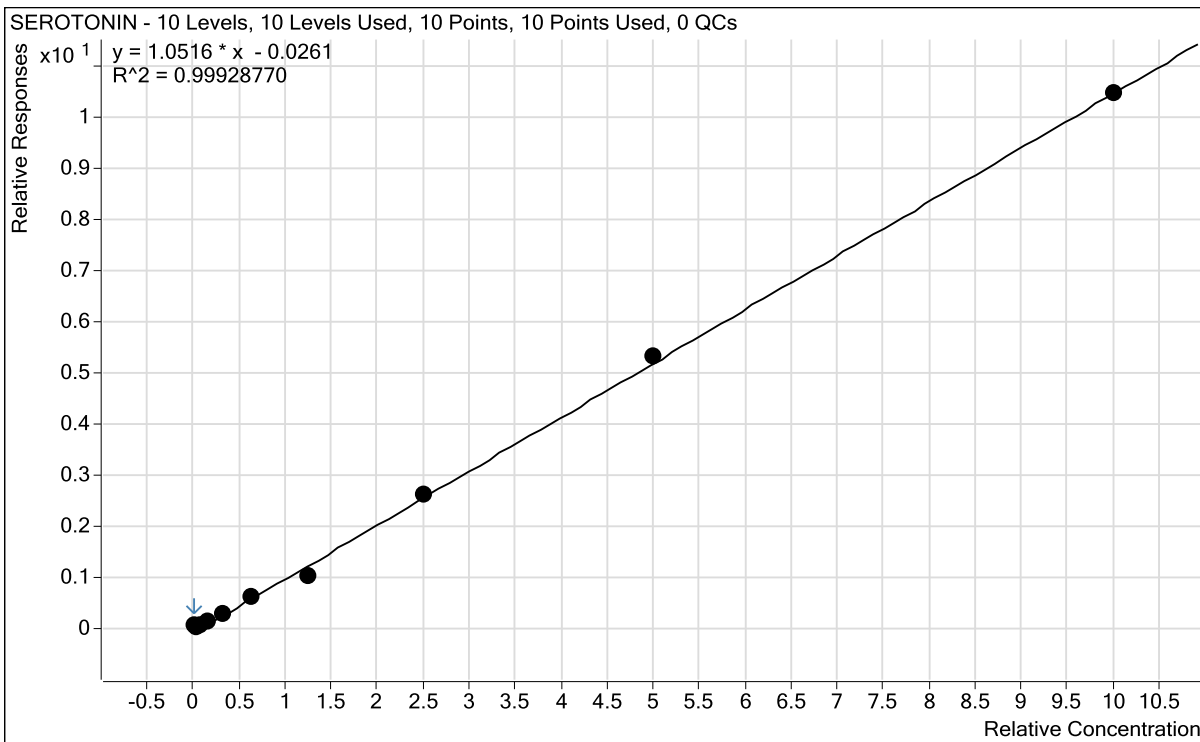
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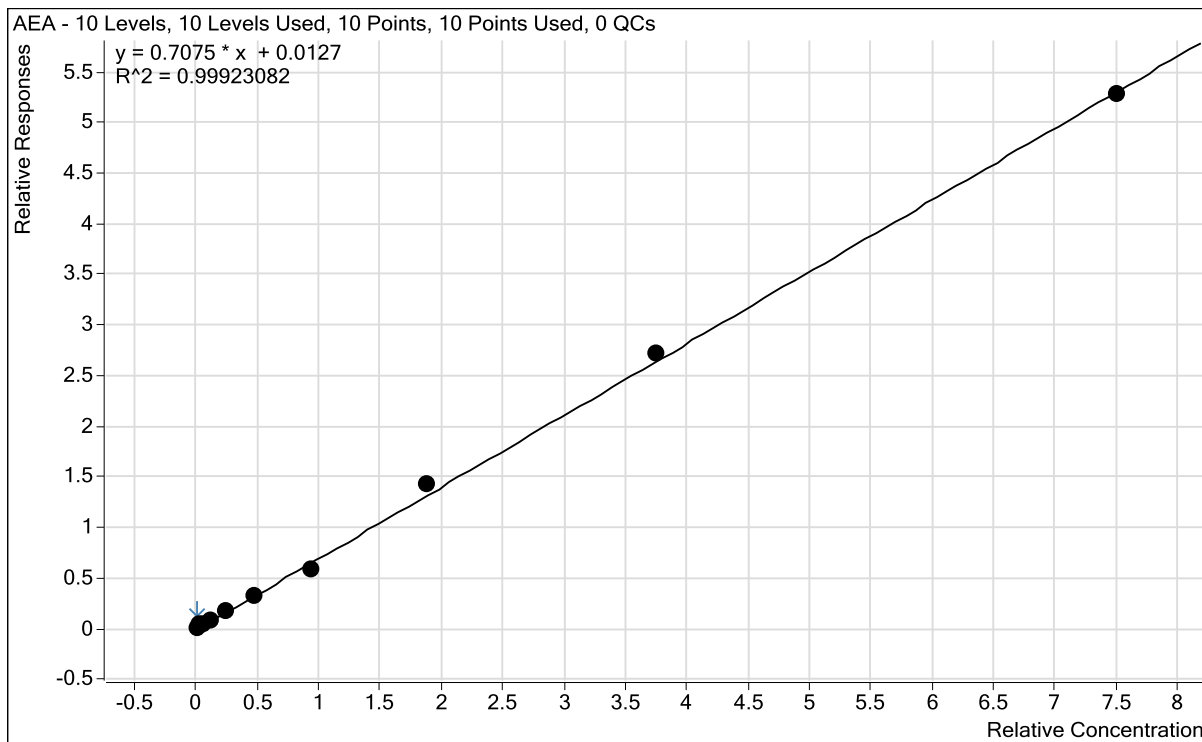
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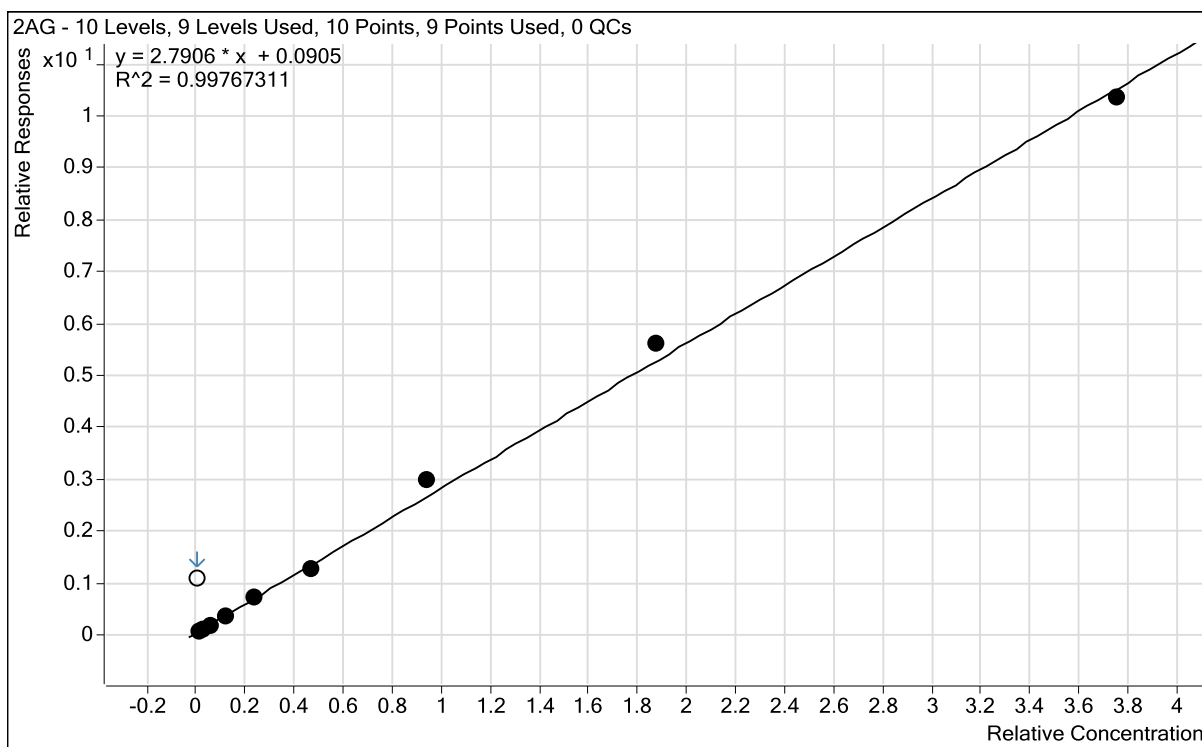
C)



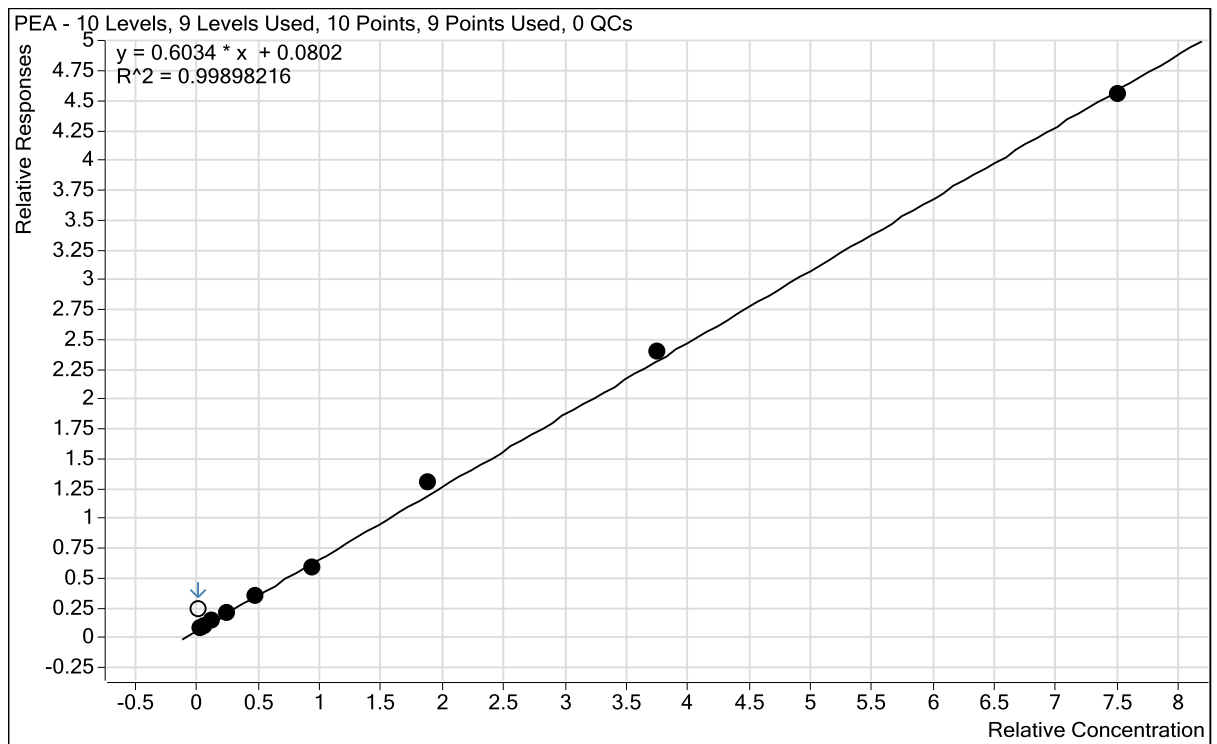
D)



E)



F)



G)

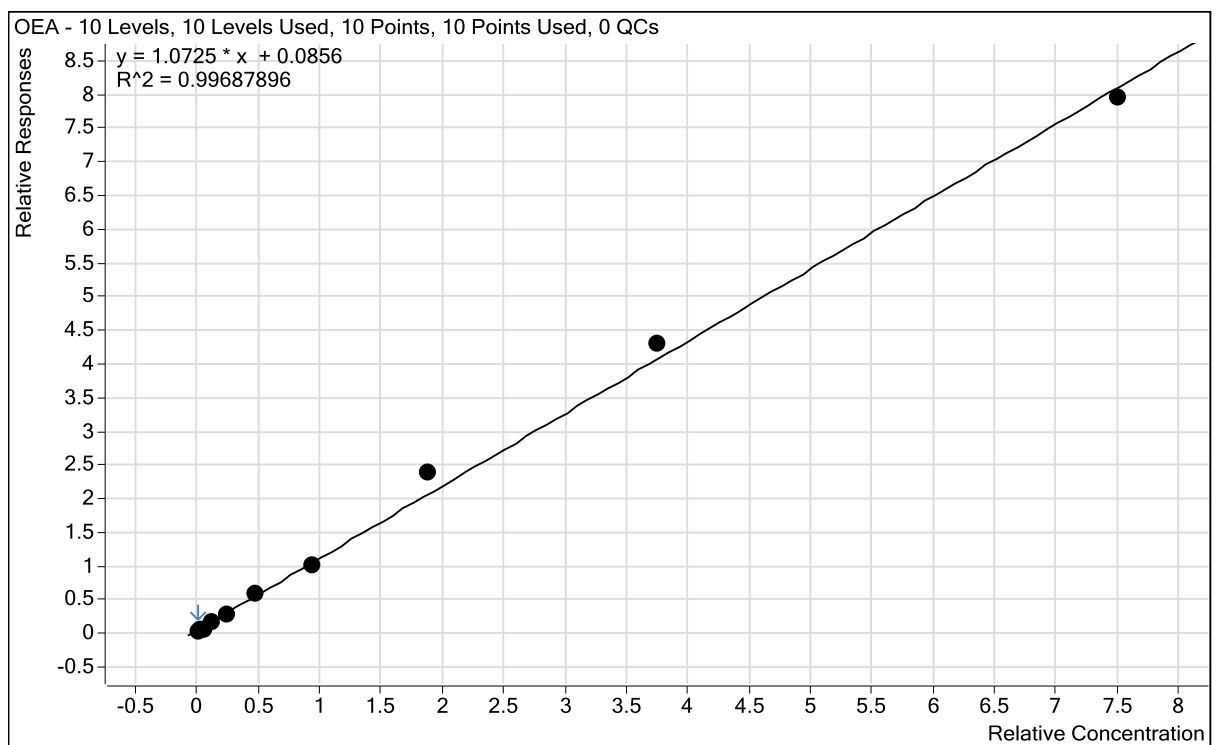
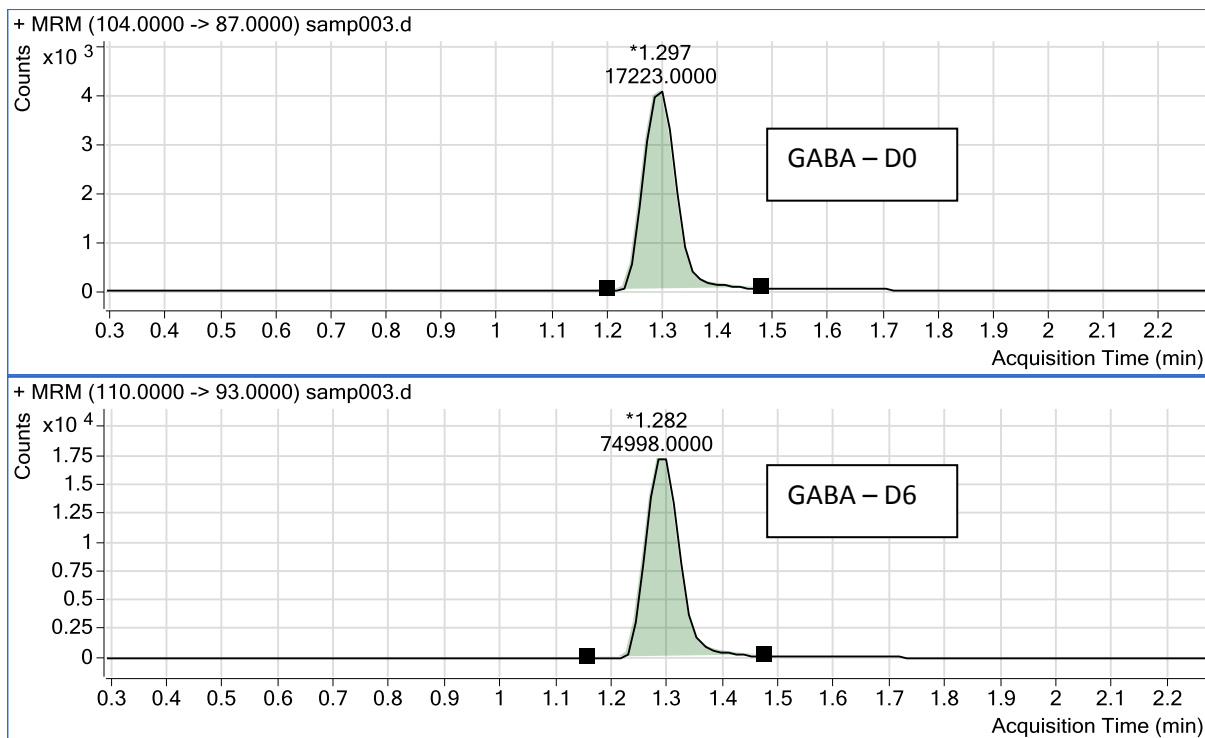
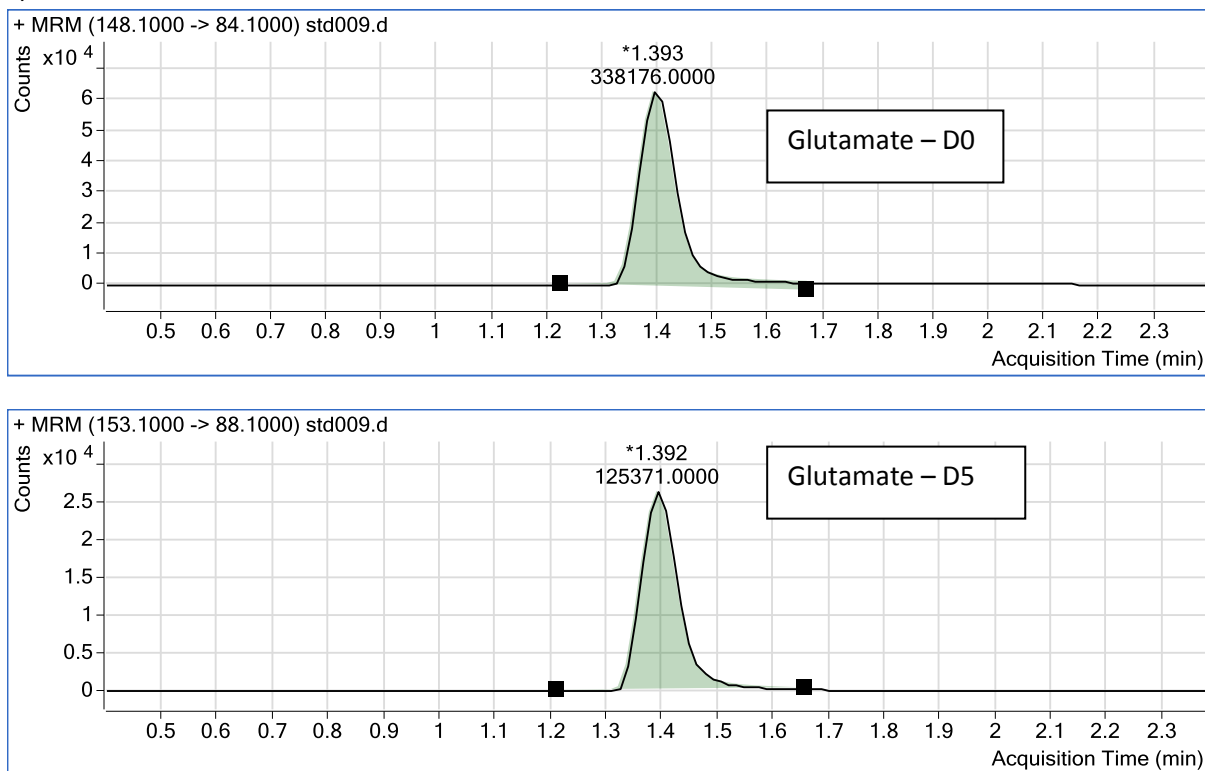


Figure 3.2: Sample 10-point calibration curves constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard for neurotransmitters and endocannabinoids A) GABA B) Glutamate C) Serotonin D) AEA E) 2-AG F) PEA G) OEA; Relative response on the y-axis is the ratio of peak area of undeuterated analyte to peak area of deuterated analyte; whereas, relative concentration on the x-axis is the ratio of amount in ng of undeuterated analyte to amount in ng of deuterated analyte.

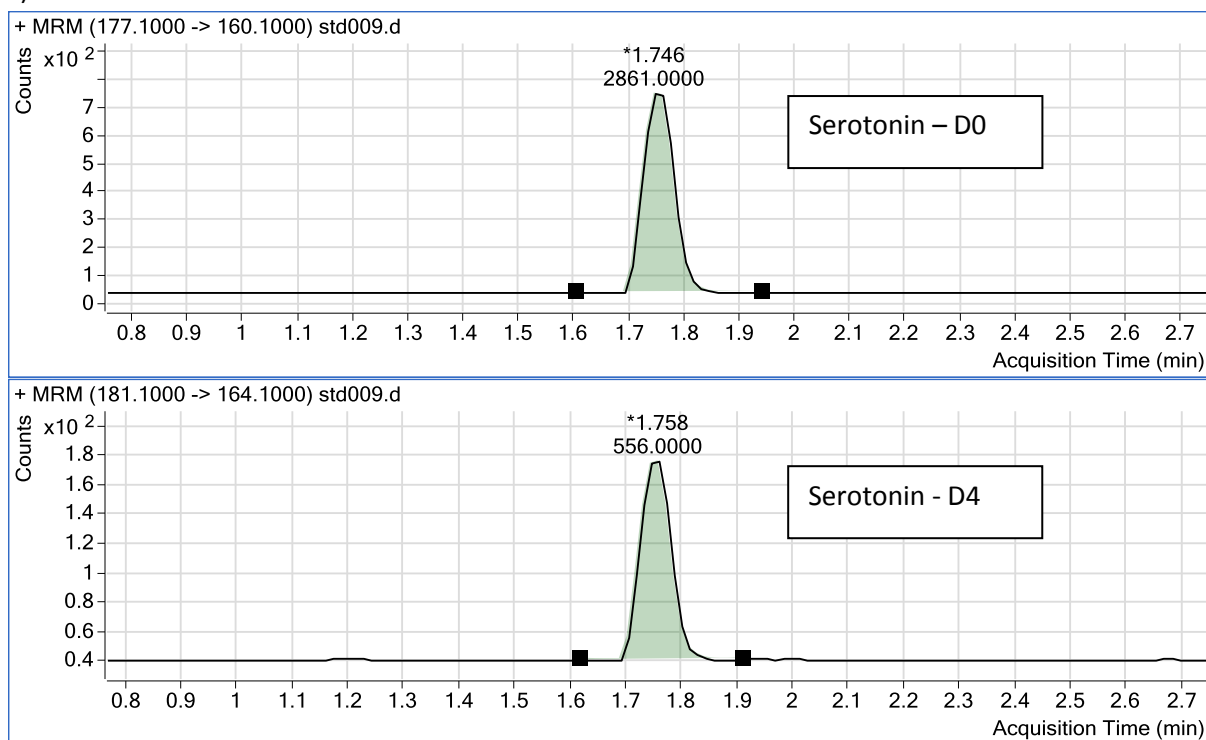
A)



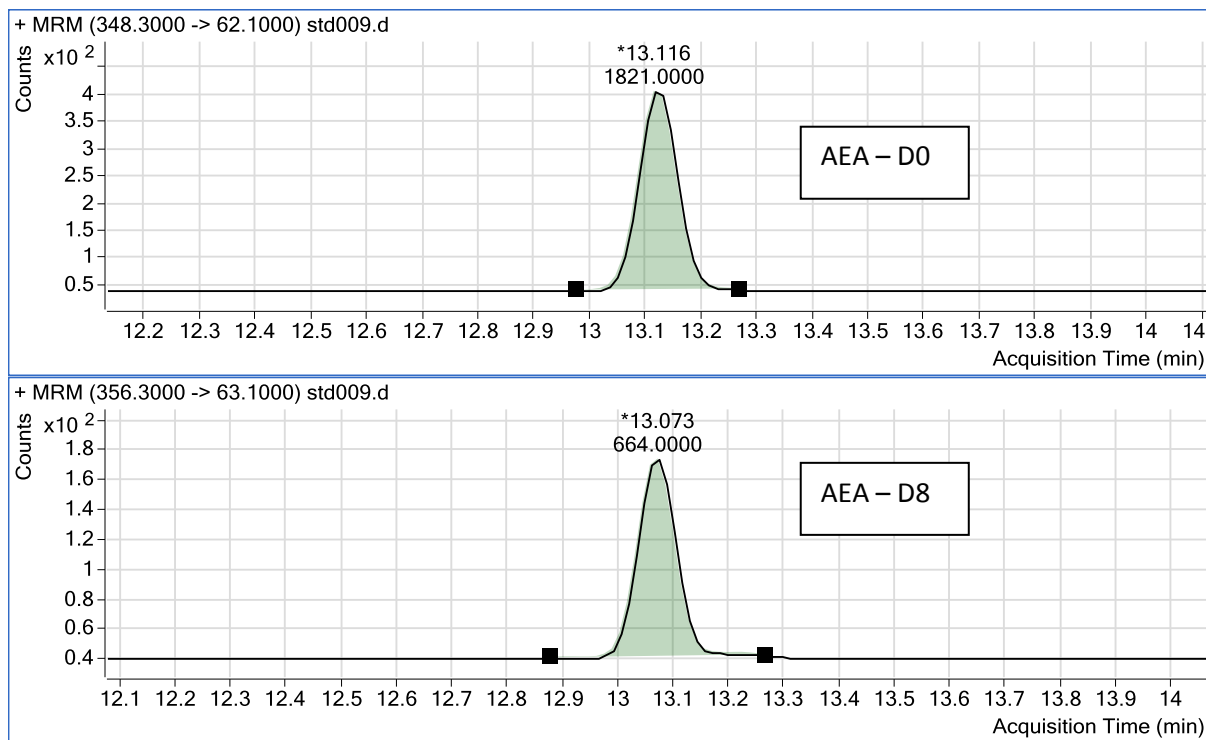
B)



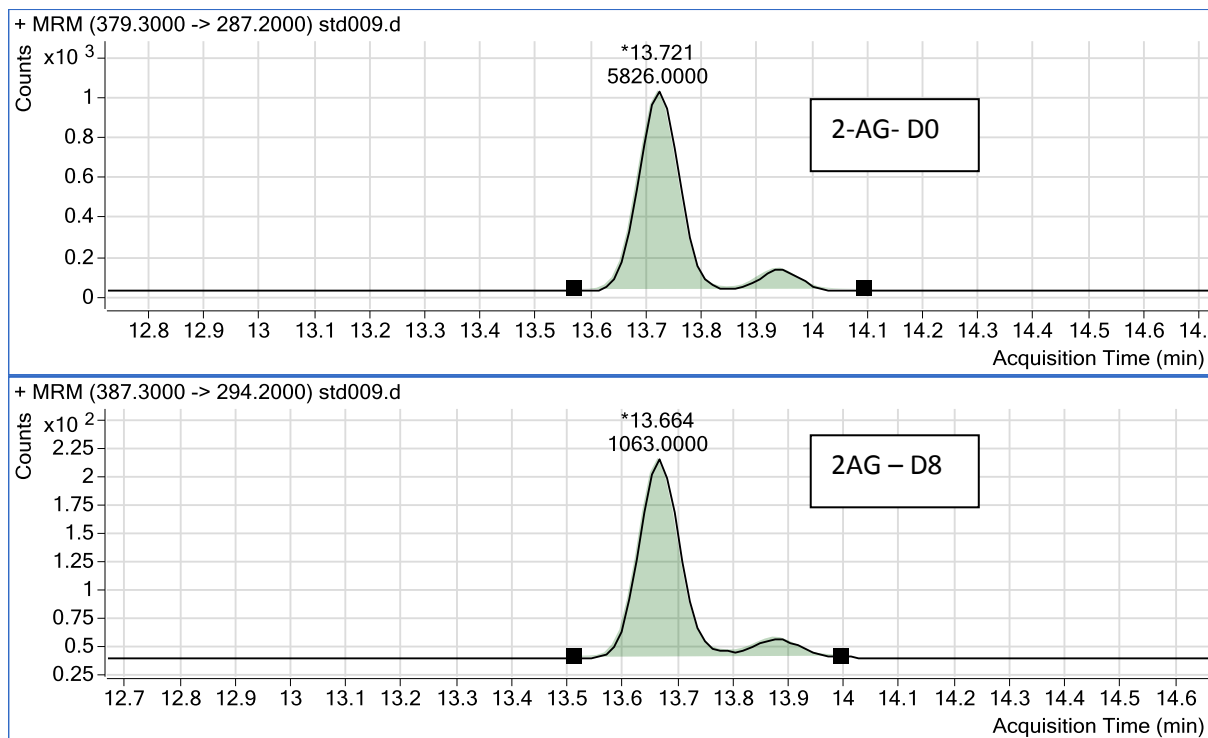
C)



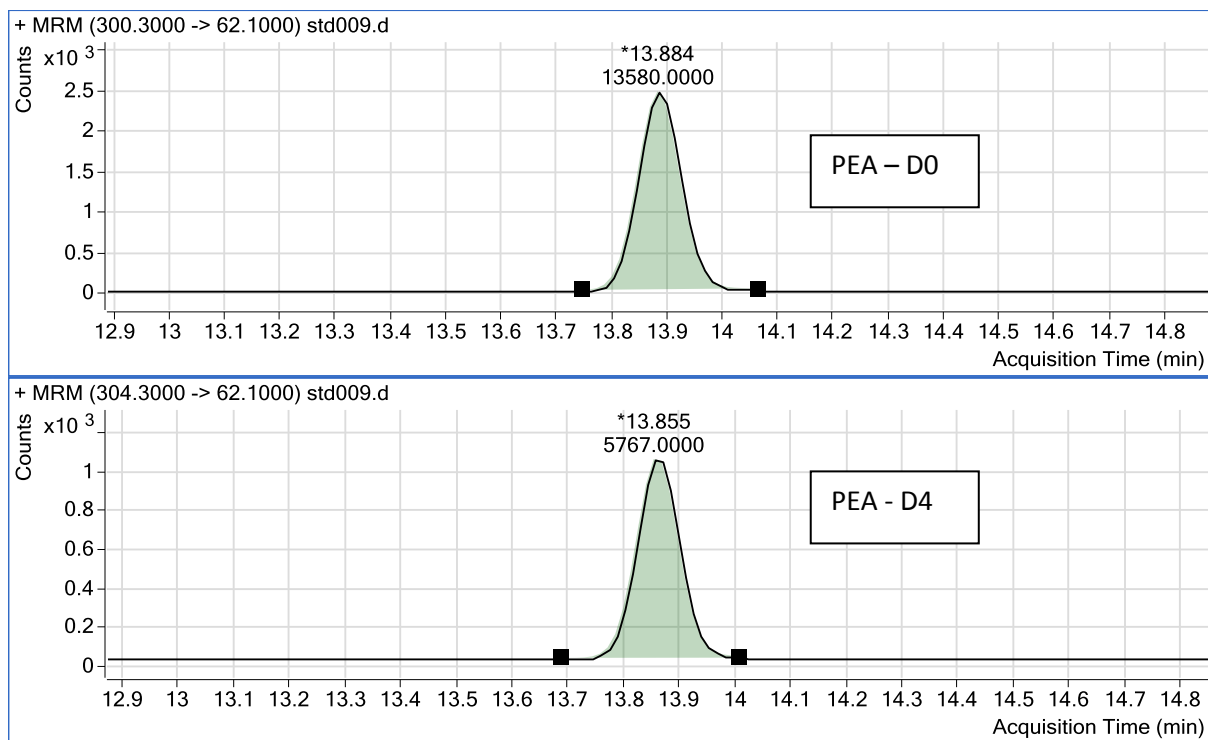
D)



E)



F)



G)

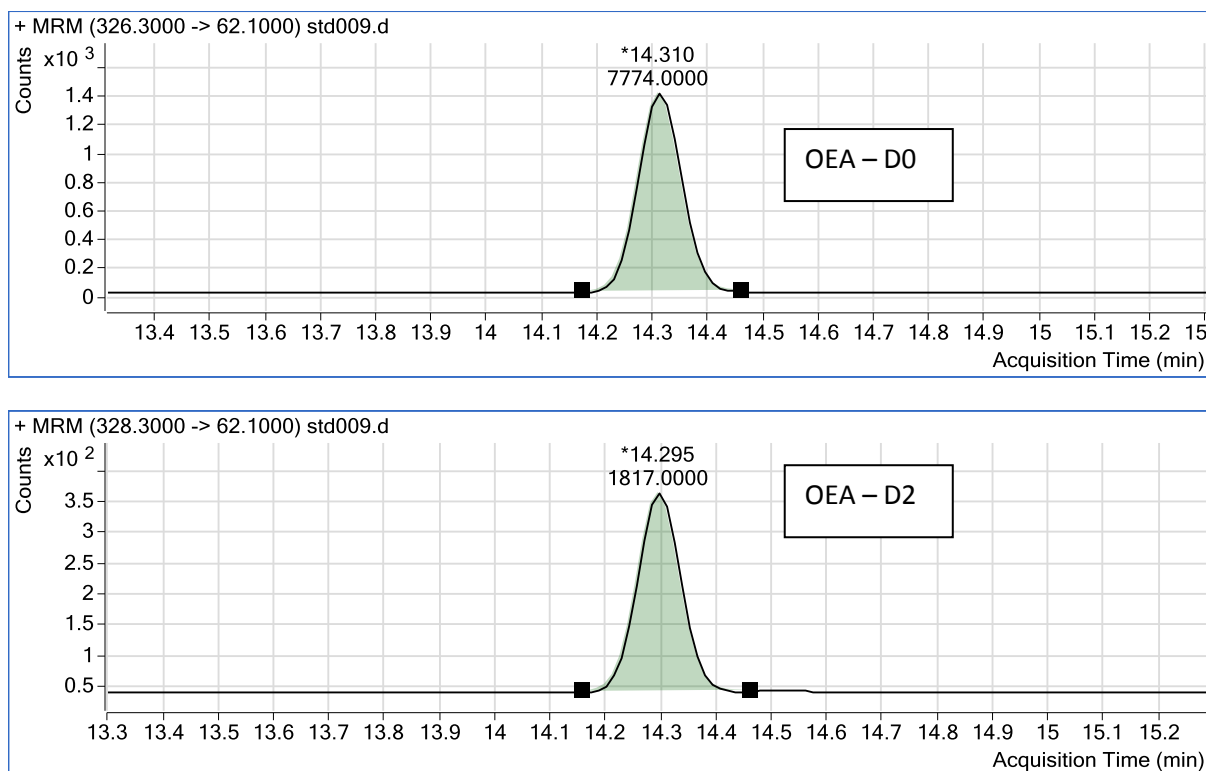


Figure 3.3: MRM spectra and mass to charge ratios of each analyte of interest and its corresponding internal standard A) GABA B) Glutamate C) Serotonin D) AEA E) 2-AG F) PEA G) OEA

3.2.12. Data analysis

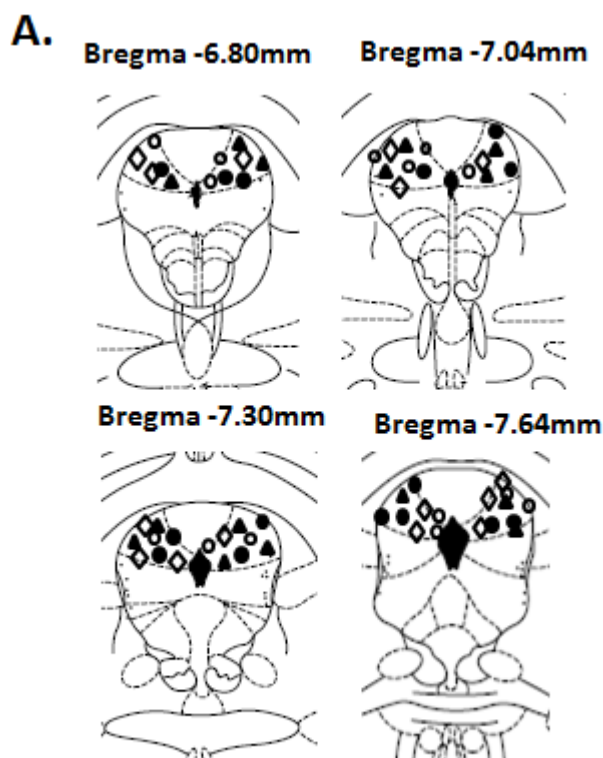
The SPSS statistical package (IBM SPSS v22.0 for Windows; SPSS, Inc., USA) was used to analyze all data. Shapiro–Wilk test confirmed that all data with the exception of defecation data were normally distributed. Levene’s test for equality of variance confirmed that the data was homogenous ($P > 0.05$). If not homogenous then progressed to parametric statistics provided that (a) the dependent variable was continuous and (b) largest variance is no more than 3 times the smallest (Dean and Voss, 1999). Analysis of formalin test timecourse data was carried out by two-way repeated measures ANOVA followed by Fisher's LSD post hoc test. Analysis of mRNA data, and general/locomotor behaviours during the pre-formalin and post-formalin trials were carried out using two-way ANOVA followed by Fisher's LSD post hoc test where appropriate. Three-way ANOVA was used to analyse the effects of pharmacological modulation on neurotransmitters and endocannabinoids in the dorsal horn of the spinal cord and Two-way ANOVA in the RVM followed by Fisher's LSD post hoc test where appropriate. Defecation (pellet number) data were analysed by a nonparametric

Kruskal-Wallis test followed by pairwise comparison with Mann-Whitney U tests. Data were considered significant when $P < 0.05$. Results are expressed as group mean \pm standard error of the mean (SEM) for parametric data and median (with interquartile range) for nonparametric data.

3.3 Results

3.3.1 Histological verification of injector site location

Following sectioning of the PAG, injection sites were verified under a light microscope, and only those rats with injections placed within the borders of both the left and right DLPAG in SD and WKY rats were included in the final analyses. 21/33 and 25/34 of the injections were placed within the borders of both the right and left DLPAG of SD and WKY rats, respectively (Fig. 3.4), with the remaining 12/33 and 9/34 of rats having one or both cannulae positioned in the LPAG, or outside of the PAG in the deep white layer of the superior colliculus. (Refer to Table 3.1 for final n numbers).



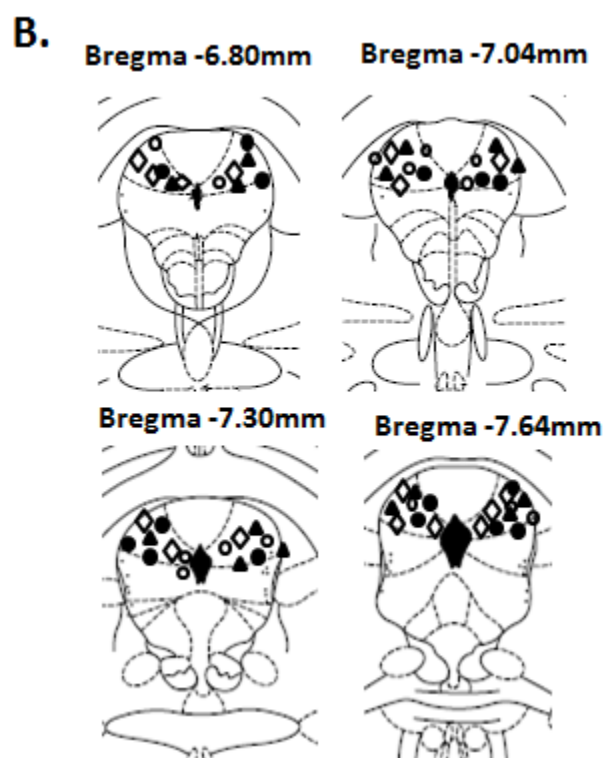


Figure 3.4: Schematic representation of vehicle (○) or capsaicin (▲) or 5'-IRTX (◆) or a combination of capsaicin and 5'-IRTX (●) injections into DLPAG for (A) SD and (B) WKY rats.

3.3.2 Effects of bilateral intra-DLPAG administration of a TRPV1 receptor agonist, antagonist, or their combination, on general exploratory/locomotor behaviours during the pre-formalin trial

Intra-DLPAG administration of capsaicin or 5'-IRTX, or the combination of both, had no significant effect on the distance moved, grooming or defecation when compared with vehicle-treated SD or WKY controls during the 10 min pre-formalin trial. Two-way ANOVA revealed significant strain effect ($P < 0.01$) on rearing activity during the pre-formalin trial period. Capsaicin- and 5'-IRTX-treated WKY rats exhibited lower rearing activity, compared with SD counterparts (Table 3.3, $P < 0.05$ WKY-CAP vs SD-CAP, $P < 0.05$ WKY-5'-IRTX vs SD-5'-IRTX), and similar trends were observed in WKY rats receiving vehicle or the combination of CAP and 5'-IRTX, compared with SD counterparts.

Group	Distance moved(cm)	grooming(s)	rearing(s)	Defecation (Pellet number)
SD-Vehicle	1966.3±146.8	5.3±1.7	45.2±15.3	0 (0-1)
SD-CAP	1964.7±159.3	23.6±14.5	53.8±14.9	1 (0-1)
SD-5'-I-RTX	1847.2±111	10.2±2.8	55.5±13.1	0 (0-1)
SD-CAP+5'-IRTX	1414.2±186.3	3.4±1.6	41±15.5	0
WKY-Vehicle	1525.3±270.4	13.5±3.5	18.3±4.1	0 (0-1)
WKY-CAP	1843.9±202.3	11.3±4.3	22.6±7.1*	0 (0-1)
WKY-5'-I-RTX	2165.1±376.7	13.4±9.3	23.9±8.5 [#]	0
WKY-CAP+5'-IRTX	1889.8±239.7	5.1±1.6	26.5±7.3	0 (0-1)

Table 3.3: Effects of intra-DLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,35}=0.108$, $P = 0.785$); drug treatment ($F_{3,35}=0.805$, $P = 0.5$) and strain X drug treatment interaction ($F_{1,35}= 1.371$, $P = 0.268$); Grooming: Two-way ANOVA effects of strain ($F_{1,35}=0.002$, $P = 0.967$); drug treatment ($F_{3,35}= 1.353$, $P = 0.273$) and strain X drug treatment interaction ($F_{1,35}=0.881$, $P = 0.460$); Rearing: Two-way ANOVA effects of strain ($F_{1,35}=11.792$, $P < 0.01$); drug treatment ($F_{3,35}=0.243$, $P = 0.866$) and strain X drug treatment interaction ($F_{1,35}= 0.272$, $P = 0.845$)

followed by Fisher's LSD post hoc test ($*P < 0.05$, SD-CAP; $^{\#}P < 0.05$ SD-5'-IRTX); Defecation: Kruskal Wallis variance of analysis by rank ($X^2 = 3.174$, $P = 0.868$). Data are expressed as mean \pm SEM for parametric data and median (interquartile range) for nonparametric data (n = 5 - 7 rats per group).

3.3.3 Effects of intra-DLPAG administration of capsaicin, 5'-IRTX or their combination on formalin-evoked nociceptive behaviour and on general exploratory/locomotor behaviours during the formalin test in SD and WKY rats.

Two-way repeated measures ANOVA revealed a significant time ($P < 0.001$), time X drug treatment interaction ($P < 0.05$), and time X strain interaction ($P < 0.001$) effect on formalin-evoked nociceptive behaviour. WKY rats that received intra-DLPAG vehicle exhibited higher nociceptive behaviour, compared with SD counterparts (Fig 3.5a WKY-Vehicle vs SD-Vehicle, $P < 0.001$), confirming the hyperalgesic phenotype in the WKY strain. In SD rats, intra-DLPAG administration of capsaicin (Fig 3.5b SD-CAP vs SD-Vehicle, $P < 0.05$ $P < 0.01$) or 5'-IRTX (Fig 3.5b SD-5'-IRTX vs SD-Vehicle, $P < 0.05$, $P < 0.01$, $P < 0.001$) significantly increased formalin-evoked nociceptive behaviour, in the second phase of the formalin trial, compared with vehicle-treated SD controls. Interestingly, these effects of capsaicin and 5'-IRTX were not observed in WKY rats (Fig 3.5c). Co-administration of capsaicin with 5'-IRTX had no effect on formalin-evoked nociceptive behaviour when compared with vehicle drug treatment in either SD or WKY rats (Fig 3.5b, Fig 3.5c). Two-way ANOVA revealed a significant strain effect ($P < 0.001$) on the distance moved during the post formalin trial. WKY rats, irrespective of intra-DLPAG drug treatment, exhibited a decrease in distance moved during the formalin test when compared to SD counterparts (Table 3.4, $P < 0.01$, SD-Vehicle vs WKY-Vehicle; $P < 0.05$, SD-CAP vs WKY-CAP; $P < 0.001$ SD-5'-IRTX vs WKY-5'-IRTX; $P < 0.01$ SD-CAP+5'-I-RTX vs WKY-CAP+5'-I-RTX). No effect of strain or drug treatment was observed on rearing, grooming or defecation over this period (Table 3.4). Two-way ANOVA indicated that there is a difference between SD and WKY rats overall in the change in hind paw diameter, but the difference did not reach statistical significance in the post hoc test (Fig 3.6). There were no significant effects of drug treatment on hind paw diameter in either strain (Fig 3.6).

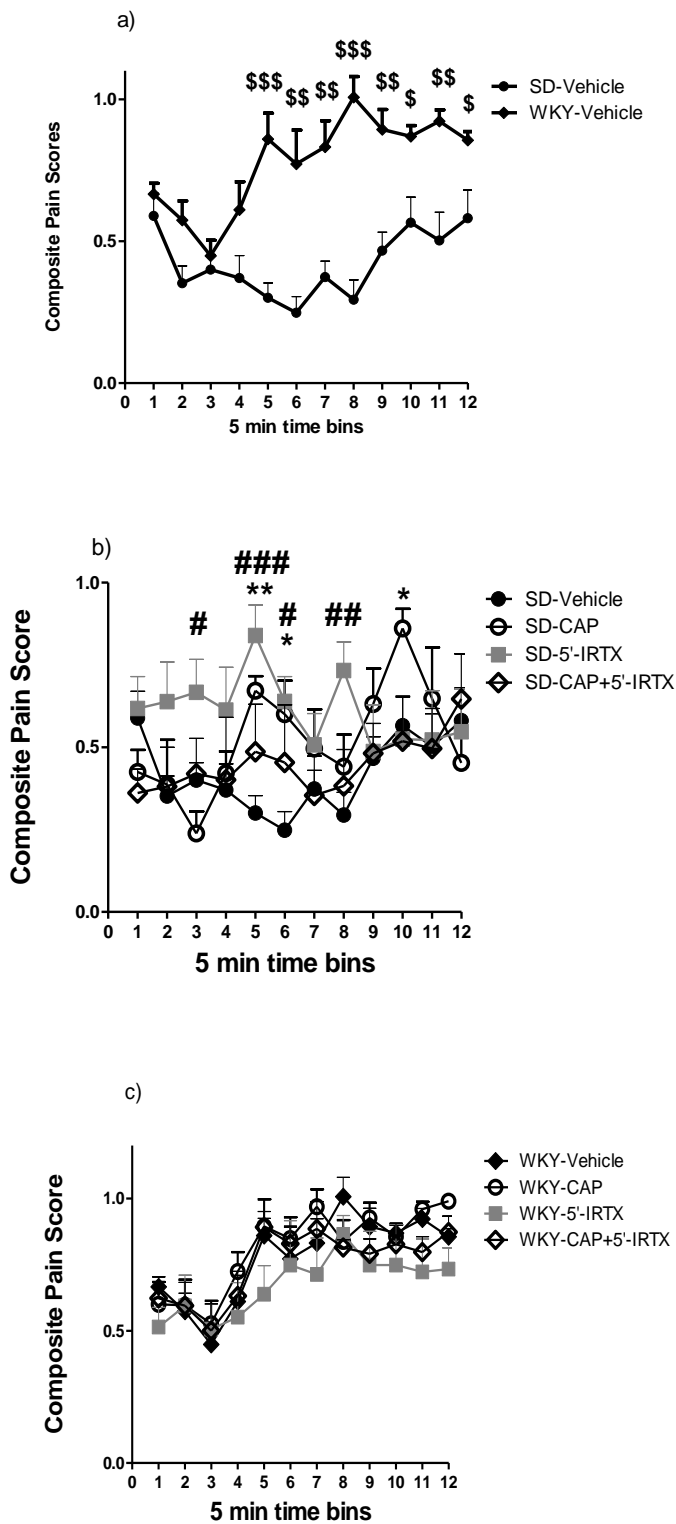


Figure 3.5: (a) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving intra-DLPAG administration of vehicle. Intra-DLPAG administration of either the TRPV1 agonist capsaicin or the TRPV1 antagonist 5'-Iodoresiniferatoxin (5'-IRTX) significantly increased formalin-evoked nociceptive behaviour in (b) SD rats, but not in (c) WKY rats. Two-way repeated measures ANOVA, time: $F_{11,418} = 9.038$, $P < 0.001$; time \times strain: $F_{11,418} = 3.927$, $P < 0.001$; time \times drug treatment: $F_{33,418} = 1.564$, $P < 0.05$; and time \times strain \times drug treatment interaction: $F_{33,418} = 1.212$, $P = 0.199$) followed by Fisher's LSD post hoc test (Fig 2a $\$P < 0.05$, $\$\$P < 0.01$, $\$$$P < 0.001$, WKY-Vehicle vs SD-Vehicle; Fig 2b $*P < 0.05$,

** $P < 0.01$, SD-CAP vs SD-Vehicle; # $P < 0.05$, SD-5'-IRTX vs SD-Vehicle). Data are expressed as mean \pm SEM (n = 5 -7 rats per group).

Group	Distance moved(cm)	Grooming(s)	Rearing(s)	Defecation (pellet number)
SD-Vehicle	5474.0 \pm 857.5	13.5 \pm 6.7	3.8 \pm 0.2	1 (0-3)
SD-Capsaicin	5257.5 \pm 1051.2	4.2 \pm 2.0	3.4 \pm 0.1	0 (0-1)
SD-5'-I-RTX	6001.8 \pm 814.1	4.2 \pm 3.9	18.6 \pm 12.2	1 (1-4)
SD- CAP+5'-IRTX	6127.2 \pm 851.1	5.6 \pm 5.0	15.2 \pm 13.6	1 (0-2)
WKY-Vehicle	2916.1 \pm 251.4 ^{\$\$}	1.3 \pm 0.8	3.5 \pm 1.0	2 (0-3)
WKY-Capsaicin	3018.5 \pm 205.8 [*]	6.1 \pm 1.8	3.5 \pm 1.4	1 (0-1)
WKY-5'-I-RTX	2863.9 \pm 215.3 ^{###}	3.4 \pm 2.4	10.6 \pm 6.34	0.5 (0-2)
WKY-CAP+5'-IRTX	3393.1 \pm 300.8 ⁺⁺	2.6 \pm 1.2	4.0 \pm 1.1	1 (1-3)

Table 3.4: Effects of intra-DLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation during the 60 min formalin trial in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,35}=41.543$, $P < 0.001$); drug treatment ($F_{3,35}=0.452$, $P = 0.717$) and strain X drug treatment interaction ($F_{1,35}= 0.202$, $P = 0.894$); Grooming: Two-way ANOVA effects of strain ($F_{1,35}=2.303$, $P = 0.139$); drug treatment ($F_{3,35}= 0.53$, $P = 0.665$) and strain X drug treatment interaction ($F_{1,35}=1.776$, $P = 0.172$); Rearing: Two-way ANOVA effects of strain ($F_{1,35}=1.032$, $P = 0.317$); drug treatment ($F_{3,35}=1.253$, $P = 0.307$) and strain X drug treatment interaction ($F_{1,35}= 0.358$, $P = 0.784$) followed by Fisher's LSD post hoc test (^{\$\$} $P < 0.01$, SD-Vehicle; ^{*} $P < 0.05$, SD-CAP; ^{###} $P < 0.001$ SD-5'-IRTX; ⁺⁺ $P < 0.01$ SD- CAP+5'-I-RTX); Defecation: Kruskal Wallis variance of analysis by rank ($X^2 = 2.38$, $P = 0.936$). Data are expressed as mean \pm SEM for parametric data and median (interquartile range) for nonparametric data (n = 5 - 7 rats per group).

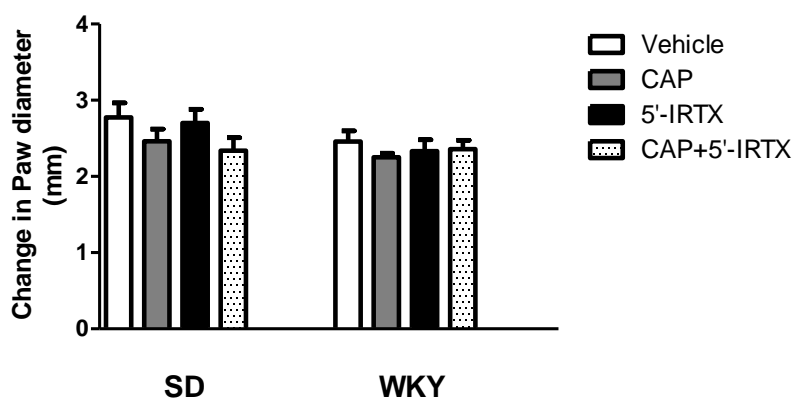


Figure 3.6: Difference in paw diameter (mm) pre- and post-formalin injection. Two-way ANOVA (effects of strain ($F_{1,35} = 4.289$, $P < 0.05$); drug treatment ($F_{3,35} = 1.496$, $P = 0.233$) and strain X drug treatment interaction ($F_{1,35} = 0.648$, $P = 0.589$) followed by Fisher's LSD post hoc test. Data are means \pm S.E.M, $n=5-7$. (SD: Sprague-Dawley, WKY:Wistar-Kyoto).

3.3.4 Effects of intra-DLPAG administration of vehicle, capsaicin, 5'-IRTX and the combination of capsaicin and 5'-IRTX on levels of neurotransmitters and endocannabinoids/N-acylethanolamines in the RVM of formalin-injected SD and WKY rats.

Intra-DLPAG administration of vehicle, capsaicin, 5'-IRTX or their combination had no effect on the neurotransmitter levels (GABA, glutamate and serotonin) or endocannabinoid/N-acylethanolamine levels (AEA, 2-AG, OEA and PEA) (Table 3.5) in the RVM of SD or WKY rats 60 min post formalin administration. Two-way ANOVA revealed significant strain differences in GABA levels and serotonin but differences between SD and WKY rats did not reach statistical significance in pairwise *Post hoc* tests, apart from an increase in the levels of serotonin in WKY rats following intra-DLPAG injection of the combination of capsaicin and 5'-IRTX, when compared to SD counterparts (Table 3.5, $P < 0.05$, SD-CAP+5'-IRTX vs WKY-CAP+5'-IRTX).

Group	GABA μmol/g tissue weight	Glutamate μmol/g tissue weight	Serotonin nmol/g tissue weight	AEA pmol/g tissue weight	2-AG nmol/g tissue weight	OEA nmol/g tissue weight	PEA nmol/g tissue weight
SD-Vehicle	9.8±1.6	36.7±4.8	2.7±0.3	88.6±44.0	62.1±8.0	1.9±0.2	1.4±0.2
SD-Capsaicin	11.2±2.6	42.1±5.4	2.7±0.1	162.0±49.1	91.2±16.4	2.8±0.3	2.1±0.4
SD-5'-I-RTX	11.0±1.5	38.1±4.1	2.8±0.2	51.1±9.2	59.7±6.0	1.9±0.2	1.3±0.1
SD- CAP+5'-IRTX	10.9±2.0	58.7±18.3	2.7±0.1	144.7±61.4	111.7±43.7	3.0±1.0	2.3±0.8
WKY-Vehicle	6.7±0.9	39.0±6.6	2.9±0.1	55.2±11.3	73.2±29.3	2.9±0.7	1.9±0.6
WKY-Capsaicin	7.7±1.0	64.5±17.4	2.6±0.2	63.5±12.2	84.9±20.7	3.4±0.8	2.3±0.7
WKY-5'-I-RTX	6.6±0.5	28.4±2.1	2.8±0.2	73.9±14.6	42.4±4.2	2.3±0.2	1.4±0.1
WKY- CAP+5'-IRTX	7.0±1.0	30.0±5.8	3.0±0.2 ⁺	45.7±6.5	42.8±3.7	2.2±0.3	1.2±0.1

Table 3.5: Effects of intra-DLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on neurotransmitter levels and endocannabinoid/ N-acylethanolamine levels in the RVM post-formalin trial in SD and WKY rats. GABA: Two-way ANOVA effects of strain ($F_{1,44}=5.329$, $P < 0.05$); drug treatment ($F_{3,44}=0.086$, $P = 0.967$) and strain X drug treatment interaction ($F_{3,44}= 0.495$, $P = 0.688$); Glutamate: Two-way ANOVA effects of strain ($F_{1,45}=0.226$, $P = 0.637$); drug treatment ($F_{3,45}= 1.38$, $P = 0.263$) and strain X drug treatment interaction ($F_{1,45}=2.17$, $P = 0.107$); Serotonin: Two-way ANOVA effects of strain ($F_{1,46}=4.744$, $P < 0.05$); drug treatment ($F_{3,46}= 0.711$, $P = 0.551$) and strain X drug treatment interaction ($F_{1,46}=1.083$, $P = 0.368$);); AEA: Two-way ANOVA effects of strain ($F_{1,45}= 1.727$, $P = 0.197$); drug treatment ($F_{3,45}=1.57$, $P = 0.213$) and strain X drug treatment interaction ($F_{3,45}=0.786$, $P = 0.509$); 2-AG: Two-way ANOVA effects of strain ($F_{1,46}= 1.631$, $P = 0.209$); drug treatment ($F_{3,46}=0.917$, $P = 0.442$); and strain X drug treatment interaction ($F_{3,46}=1.22$, $P = 0.315$); OEA: Two-way ANOVA effects of strain ($F_{1,46}= 0.64$, $P = 0.429$); drug treatment ($F_{3,46}=0.972$, $P = 0.416$) and strain X drug treatment interaction ($F_{3,46}=1.022$, $P = 0.393$); PEA: Two-way ANOVA effects of strain ($F_{1,46}=0.083$, $P = 0.775$); drug treatment ($F_{3,46}= 0.994$, $P = 0.406$) and strain X drug treatment interaction ($F_{3,46}=0.989$, $P = 0.408$); followed by Fisher's LSD post hoc test; (⁺ $P < 0.05$ vs SD-CAP+5'-IRTX); Data are expressed as mean ± SEM (n = 5 - 7 rats per group).

3.3.5 Effects of intra-DLPAG administration of vehicle, capsaicin, 5'-IRTX and the combination of capsaicin and 5'-IRTX on neurotransmitters and endocannabinoids/N-acylethanolamines in the dorsal horn of the spinal cord (ipsilateral and contralateral sides) of formalin-injected SD rats and WKY rats.

Intra-DLPAG administration of vehicle, capsaicin, 5'-IRTX and combination of capsaicin and 5'-IRTX had no effect on the levels of neurotransmitters (GABA, Glutamate and Serotonin) or endocannabinoids/N-acylethanolamines (AEA, 2-AG and OEA) in either SD and WKY formalin-treated rats on the contralateral side of the dorsal horn of the spinal cord (Table 3.6). Intra-DLPAG administration of vehicle, capsaicin, 5'-IRTX and combination of capsaicin and 5'-IRTX had no effect on the levels of neurotransmitters (GABA and serotonin) or endocannabinoids/N-acylethanolamines (AEA, 2-AG) in either SD or WKY formalin-treated rats on the ipsilateral side of the dorsal horn of the spinal cord (Table 3.6). Three-way ANOVA revealed significant differences between the ipsilateral and contralateral sides of dorsal horn of spinal cord for the neurotransmitters (glutamate and serotonin) and endocannabinoids/N-acylethanolamines (2-AG, OEA and PEA) (Table 3.6 $P < 0.05$, $P < 0.01$, $P < 0.001$ vs respective ipsilateral counterparts) levels. Three-way ANOVA revealed significant strain differences in serotonin and PEA levels between the SD and WKY rats, but these did not reach statistical significance in pairwise *Post hoc* tests. Intra-DLPAG administration of both capsaicin and 5'-IRTX in combination decreased the levels of PEA, OEA and Glutamate on the ipsilateral side of the dorsal horn of spinal cord in WKY rats but not in SD rats (Table 3.6, $P < 0.05$, WKY-Cap+5'-IRTX vs WKY-Vehicle). This difference was not observed for the contralateral side of the dorsal horn of the spinal cord (Table 3.6).

Group	GABA µmol/g tissue weight	Glutamate µmol/g tissue weight	Serotonin nmol/g tissue weight	AEA pmol/g tissue weight	2-AG nmol/g tissue weight	OEA nmol/g tissue weight	PEA nmol/g tissue weight
Contralateralside of DHSC							
SD-Vehicle	6.5±1.6	26.8±8.7	7.6±1.6	68.9±4.6	53.4±15.8	1.9±0.3	1.5±0.2
SD-Capsaicin	7.7±0.5	35.5±5.3	9.6±0.7	91.6±18.6	80.4±18.8	2.5±0.1	2.0±0.2
SD-5'-I-RTX	7.2±1.5	25.4±6.4	8.3±1.2	80.0±12.1	76.2±24.3	2.1±0.4	1.7±0.3
SD- CAP+5'-IRTX	7.9±2.6	30.0±9.8	9.2±1.6	96.3±21.0	84.0±37.1	2.1±0.2	1.9±0.3
WKY-Vehicle	6.2±1.2	28.6±8.2	6.3±1.4	74.2±16.4	64.0±21.9	2.0±0.4	1.5±0.3
WKY-Capsaicin	6.0±0.9	24.3±3.3	6.2±0.7	67.7±11.9	63.9±11.8	2.0±0.4	1.5±0.3
WKY-5'-IRTX	5.9±0.2	20.1±2.4	5.4±0.9	86.3±7.1	66.9±12.9	2.2±0.3	1.7±0.1
WKY- CAP+5'-IRTX	4.0±1.0	14.6±5.1	4.0±0.8	63.3±18.8	41.5±20.9	1.4±0.3	1.1±0.3
Ipsilateralside of DHSC							
SD-Vehicle	6.9±0.8	30.4±3.1	8.5±1.9	62.6±5.3	26.7±4.8	2.3±0.2	1.7±0.2 [£]
SD-Capsaicin	6.0±0.9	25.9±3.8	6.8±1.5	63.2±11.6	23.8±5.7 ^{££}	1.9±0.4	1.5±0.3
SD-5'-I-RTX	6.4±0.6	29.7±2.2	7.9±0.4	65.1±6.3	23.9±3.9 [£]	2.4±0.2	1.8±0.1
SD- CAP+5'-IRTX	6.2±0.5	29.8±3.7	9.1±0.7 [£]	55.0±4.0	27.7±4.2 [£]	2.4±0.3	1.7±0.2 [£]
WKY-Vehicle	5.1±0.4	26.1±3.6	6.7±0.6	55.7±4.3	19.0±1.9 [£]	2.4±0.3	1.6±0.1
WKY-Capsaicin	5.8±0.4	30.8±2.0	7.4±0.5	58.0±4.2	15.5±2.1 ^{££}	2.8±0.2	1.7±0.1
WKY-5'-I-RTX	6.1±0.2	32.9±2.1 [£]	7.4±0.7	63.0±8.2	17.8±2.7 ^{££}	2.7±0.4	1.7±0.2
WKY- CAP+5'-IRTX	6.6±0.7	39.5±3.4 ^{£££}	8.6±0.7 ^{££}	77.1±10.0	29.4±7.1	3.6±0.5 ^{+£}	2.2±0.3 ^{+£££}

Table 3.6: Effects of intra-DLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on neurotransmitter levels and endocannabinoid/N-acylethanolamine levels in the ipsilateral side of dorsal horn of spinal cord post formalin trial in SD and WKY rats. GABA: Three-way ANOVA effects of side ($F_{1,93}=0.185$, $P = 0.669$); strain ($F_{1,93}=3.114$, $P = 0.082$); drug treatment ($F_{3,93}=0.689$, $P = 0.561$); side X strain ($F_{1,93}= 0.017$, $P = 0.898$); side X drug treatment ($F_{3,93}= 1.521$, $P = 0.216$) strain X drug treatment ($F_{3,93}= 0.141$, $P = 0.935$); side X strain X drug treatment ($F_{3,93}= 0.934$, $P = 0.428$). Glutamate: Three-way ANOVA effects of side ($F_{1,91}=14.961$, $P < 0.001$); strain ($F_{1,91}=0.012$, $P = 0.913$); drug treatment ($F_{3,91}=0.099$, $P = 0.96$); side X strain ($F_{1,91}= 2.49$, $P = 0.119$); side X drug treatment ($F_{3,91}= 1.891$, $P = 0.138$);strain X drug treatment ($F_{3,91}= 0.162$, $P = 0.921$); side X strain X drug treatment ($F_{3,91}= 1.181$, $P = 0.323$). Serotonin: Three-way ANOVA effects of side ($F_{1,92}=11.498$, $P < 0.01$); strain ($F_{1,92}=6.369$, $P < 0.05$); drug treatment ($F_{3,92}=0.409$, $P = 0.747$); side X strain ($F_{1,92}= 0.531$, $P = 0.468$); side X drug treatment ($F_{3,92}= 1.997$, $P = 0.121$);strain X drug treatment ($F_{3,92}= 0.342$, $P = 0.795$); side X strain X drug treatment ($F_{3,92}= 0.692$, $P = 0.56$). AEA: Three-way ANOVA effects of side ($F_{1,92}=2.538$, $P = 0.115$); strain ($F_{1,92}=0.179$, $P = 0.674$); drug treatment ($F_{3,92}=1.316$, $P = 0.275$); side X strain ($F_{1,92}= 0.237$, $P = 0.628$); side X drug treatment ($F_{3,92}= 1.423$, $P = 0.243$);strain X drug treatment ($F_{3,92}= 0.891$, $P = 0.45$); side X strain X drug treatment ($F_{3,92}= 1.594$, $P = 0.198$). 2-AG: Three-way ANOVA effects of side ($F_{1,90}=41.96$, $P < 0.001$); strain ($F_{1,90}=1.295$, $P = 0.259$); drug treatment ($F_{3,90}=0.602$, $P = 0.616$); side X strain ($F_{1,90}= 0.059$, $P = 0.809$); side X drug treatment ($F_{3,90}= 1.7$, $P = 0.174$);strain X drug treatment ($F_{3,90}= 0.262$, $P = 0.853$); side X strain X drug treatment ($F_{3,90}= 0.539$, $P = 0.657$). PEA: Three-way ANOVA effects of side ($F_{1,92}=23.787$, $P < 0.001$); strain ($F_{1,92}=4.438$, $P < 0.05$); drug treatment ($F_{3,92}=1.11$, $P = 0.35$); side X strain ($F_{1,92}= 0.892$, $P = 0.348$); side X drug treatment ($F_{3,92}=1.854$, $P = 0.144$);strain X drug treatment ($F_{3,92}= 0.117$, $P = 0.95$); side X strain X drug treatment ($F_{3,92}= 1.01$, $P = 0.393$). OEA: Three-way ANOVA effects of side ($F_{1,92}=7.333$, $P < 0.01$); strain ($F_{1,92}=0.016$, $P = 0.9$); drug treatment ($F_{1,92}= 0.838$, $P = 0.477$); side X strain ($F_{3,92}=0.029$, $P = 0.864$); side X drug treatment ($F_{3,92}= 0.925$, $P = 0.433$); strain X drug treatment ($F_{3,92}= 0.375$, $P = 0.771$); side X strain X drug treatment ($F_{3,92}= 0.771$, $P =0.568$) followed by Fisher's LSD post hoc test; (* $P < 0.05$, vs SD-Vehicle) ($^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$, $^{\ddagger\ddagger\ddagger}P < 0.001$ vs respective contralateral counterparts) ($^{\dagger}P < 0.05$, vs WKY-Vehicle) ($^{\S}P < 0.05$, WKY-vehicle vs SD-vehicle). Data are expressed as mean \pm SEM (n = 5 - 7 rats per group).

3.3.6. Effects of intra-DLPAG administration of capsaicin or 5'-IRTX on *c-Fos* mRNA expression in the dorsal horn of the spinal cord of formalin-injected SD rats and WKY rats.

c-Fos mRNA expression was higher on the ipsilateral side when compared to the contralateral side (Fig 3.7, $P < 0.001$, vs contralateral side) of the dorsal horn of the spinal cord of formalin-injected rats. Three-way ANOVA revealed significant strain ($P < 0.001$) and strain X side interaction ($P < 0.01$) effect on *c-Fos* mRNA expression in the dorsal horn of spinal cord. WKY rats that received intra-DLPAG administration of vehicle, capsaicin or 5'-IRTX exhibited lower *c-Fos* mRNA expression on the ipsilateral side of the dorsal horn of the spinal cord, when compared to their SD counterparts (Fig 3.7, $P < 0.001$, SD-Vehicle; $P < 0.05$, SD-CAP; $P < 0.05$, SD-5'-IRTX). There were no significant effects of strain or drug treatment on *c-Fos* mRNA expression on the contralateral side of the dorsal horn of the spinal cord. We did not include the SD and WKY groups that received the combination of capsaicin + 5'-IRTX in the *c-Fos* experiments because the most significant behavioural effects were observed for the groups receiving the drugs alone.

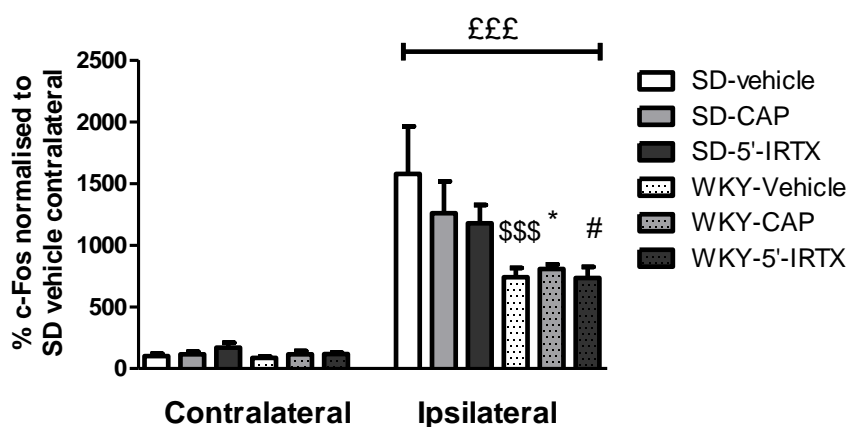


Figure 3.7: *c-Fos* mRNA expression on the ipsilateral and contralateral sides of the dorsal horn of the spinal cord after intra-DLPAG administration of vehicle, capsaicin or 5'-IRTX in SD and WKY rats. Three-way ANOVA effects of side ($F_{1,64} = 140.871$, $P < 0.001$), strain ($F_{1,64} = 14.626$, $P < 0.001$), drug treatment ($F_{2,64} = 0.337$, $P = 0.716$), side X strain interaction ($F_{1,64} = 12.492$, $P < 0.01$), side X drug treatment interaction ($F_{2,64} = 0.893$, $P = 0.416$), strain X drug treatment interaction ($F_{2,64} = 0.671$, $P = 0.515$), side X strain X drug treatment interaction ($F_{2,64} = 0.772$, $P = 0.467$); followed by Fisher's LSD post hoc test ($$$$P < 0.001$ vs SD-Vehicle; $*P < 0.05$ vs SD-CAP; $\#P < 0.05$ vs SD-5'-IRTX) ($£££P < 0.001$ vs respective ipsilateral counterpart). Data are means \pm S.E.M, $n = 5-7$. (SD: Sprague-Dawley, WKY: Wistar-Kyoto).

3.4 Discussion

The data presented in this chapter demonstrate that TRPV1 in the DLPAG column regulates formalin-evoked nociceptive behaviour differentially in SD rats versus WKY counterparts. In SD rats, intra-DLPAG administration of either capsaicin or 5'-IRTX increased formalin-evoked nociceptive behaviour. Co-administration of capsaicin and 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in SD rats. In contrast, intra-DLPAG administration of either capsaicin or 5'-IRTX had no effect on formalin-evoked behaviour in WKY rats. Intra-DLPAG administration of capsaicin, 5'-IRTX or their combination had no effects on neurotransmitters or endocannabinoid/N-acylethanolamine levels in the RVM or in the dorsal horn of the spinal cord in either strain, however, some differences were observed between discrete SD and WKY groups. *c-Fos* mRNA expression was higher on the ipsilateral side when compared to the contralateral side of the dorsal horn of the spinal cord, irrespective of drug treatment. However, WKY rats (irrespective of intra-DLPAG drug treatment) exhibited lower *c-Fos* mRNA expression on the ipsilateral side of the dorsal horn of the spinal cord, when compared to their SD counterparts.

The data presented herein demonstrate that TRPV1 in the DLPAG regulates formalin-evoked nociceptive behaviour differentially in SD rats versus WKY counterparts, and these alterations in TRPV1 expression or functionality in the DLPAG might contribute to the hyperalgesic phenotype displayed by the stress-sensitive WKY strain. In SD rats, formalin-evoked nociceptive behaviour was associated with reduced TRPV1 expression in the DLPAG (Chapter 2). WKY rats also had lower levels of TRPV1 expression in the DLPAG compared with SD rats and we hypothesized that this may explain their propensity to respond in a hyperalgesic manner to formalin injection. Moreover, in the results presented in Chapter 2 we observed a formalin-induced increase in TRPV1 expression in the DLPAG of WKY rats and hypothesized that this may represent a compensatory change in an attempt to reduce pain behaviour in the WKY strain. To further test these hypotheses, the studies described in the current chapter investigated the effects of pharmacological manipulation of TRPV1 in the DLPAG on formalin-evoked nociceptive behaviour in both strains using the TRPV1 agonist capsaicin and the TRPV1 antagonist 5'-IRTX. In SD rats, intra-DLPAG administration of either capsaicin or 5'-IRTX resulted in a pronociceptive effect, increasing formalin-evoked nociceptive behaviour. The effect of capsaicin was likely due to desensitisation of TRPV1 in the

DLPAG, given that its effects were similar to the effects of TRPV1 blockade with 5'-IRTX. Thus, these data are compatible with the idea that lower TRPV1 signalling in the DLPAG is associated with increased formalin-evoked nociceptive behaviour. Interestingly, the co-administration of capsaicin and 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in SD rats, likely due to both drugs competing dynamically for binding to TRPV1, with neither drug binding for long enough to desensitise (capsaicin) or block (5'-IRTX) the channel. In contrast to their effects in SD rats, intra-DLPAG administration of capsaicin or 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in WKY rats. One possible explanation for these findings is that the formalin-evoked increase in TRPV1 expression in WKY rats serves to counteract/oppose the desensitisation or blockade caused by capsaicin or 5'-IRTX, respectively. Drug treatment had no significant effect on locomotor activity/non-pain related behaviours in either strain when injected into the DLPAG, which suggests that the effects of the drug treatments on formalin-evoked nociceptive behaviour were specific to nociceptive processing and were not confounded by non-specific, overt effects on locomotor activity. WKY rats exhibited less rearing behaviour on exposure to the novel testing arena when compared to SD rats, as has been reported previously (Burke et al., 2010; Paré, 1994). The reduced locomotor activity of WKY rats, when compared to SD rats, during the formalin trial might relate either to the anxiodepressive phenotype of the WKY strain or alternatively/in addition, to their heightened pain-related behaviour when compared to SD counterparts.

To my knowledge, this study is the first to demonstrate a differential role of TRPV1 in the DLPAG in the regulation of formalin-evoked nociceptive behaviour in SD versus WKY rats. Previous studies have confirmed that TRPV1 within the DPAG is involved in modulating nociceptive responses to noxious heat, such that intra-DPAG capsaicin administration to SD rats produces pronociceptive effects in the rat tail flick test, an effect associated with increased ON cell activity in the RVM (McGaraughty et al., 2003). Our data demonstrating that injection of capsaicin into the DLPAG of SD rats was pronociceptive in the formalin test, supports these results and extends them to the context of inflammatory pain. Conversely, capsaicin microinjection into the DLPAG has been shown to have an antinociceptive effect in the plantar test of thermoceptive sensitivity, an effect mediated by glutamate-induced activation of mGluR1 and NMDA receptors in the DLPAG (Palazzo et al., 2002). The difference in the results from the studies of Palazzo et al and McGaraughty et al can be attributed to technical aspects of

the experiments. Palazzo et al. administered 6nmol/0.2 µl of capsaicin into the DPAG while McGaraughty et al. administered 10nmol/0.4 µl. In the study by Palazzo et al. drug was also injected at a different rate (0.1 µl/2.5s), 6 times greater than McGaraughty et al. (0.1 µl/15 s). The desensitization rate of capsaicin-sensitive neurons is concentration-dependent (Liu and Simon, 1996), thus rapid delivery of a smaller volume of a highly concentrated capsaicin solution may be sufficient to quickly desensitize a small population of PAG neurons causing the relatively small increase in withdrawal latencies. Alternatively, hyperalgesia induced by infusion of a larger volume of capsaicin into the DPAG was due to compound diffusion to other sites given the proximity of the cerebral aqueduct. This is unlikely as in McGaraughty et al. (McGaraughty et al., 2003) capsaicin had no effect after injection into the VPAG and in my studies capsaicin had different effects when administered into VLPAG which is discussed in the next chapter.

There were no effects of pharmacological modulation of TRPV1 in the DLPAG on neurotransmitters and endocannabinoid/N-acylethanolamine levels in the RVM or in the contralateral side of dorsal horn of spinal cord of SD rats. There were subtle effects of pharmacological modulation of TRPV1 in DLPAG on neurotransmitters and endocannabinoid/ N-acylethanolamine levels in the ipsilateral side of the dorsal horn of the spinal cord. In the ipsilateral side of dorsal horn of spinal cord, intra-DLPAG administration of a combination of capsaicin and 5'-IRTX decreased the levels of PEA significantly when compared to vehicle-treated rats. As stated above, the behavioural effects of intra-DLPAG administration of the capsaicin and/or 5'-ITRX were transient and were much reduced or no longer present by the end of the trial. The lack of effect of pharmacological modulation of TRPV1 in the DLPAG on levels of neurotransmitters or N-acylethanolamines suggests a lack of engagement of the descending pain pathway following TRPV1 modulation in the DLPAG. In that context, it is worth remembering that the DLPAG has no medullary projections onto the RVM directly, but indirectly influences the RVM via projection to the cuneiform nucleus which in turn connects to RVM (Holstege, 1991; Holstege and Kuypers, 1982; Keay and Bandler, 2001; Mitchell et al., 1988a, 1988b; P Redgrave et al., 1987; Peter Redgrave et al., 1987; Redgrave et al., 1988). However, one limitation of the present studies is that the neurotransmitters and endocannabinoids/N-acylethanolamines were measured at a single time point i.e. 60 mins post-formalin injection which was some time after the peak behavioural effects of the drugs and therefore it is possible that neurochemical

alterations may have been detected at an earlier time point. Furthermore, neurotransmitters and endocannabinoids are mainly released on demand dynamically and into the extracellular space, and such drug-induced effects may, therefore, be better studied using *in vivo* microdialysis over the course of the formalin trial to measure the released (signalling) pool or these neurochemicals as opposed to the tissue levels.

The data indicate that pharmacological modulation of TRPV1 in the DLPAG altered formalin-evoked nociceptive behaviour in SD rats. To further understand whether such an effect is mediated by activation of the descending inhibitory pain pathway, *c-Fos* expression was measured in the dorsal horn of the lumbar region of the spinal cord. *c-Fos* is a well-characterised index of neuronal activity (Harris, 1998). Studies have shown that *c-Fos* protein expression in the dorsal horn of the spinal cord is increased one-hour post-formalin administration (Abbadie et al., 1994, 1992; Abbadie and Besson, 1994; Gogas et al., 1991; Leah et al., 1996; Presley et al., 1990). In the present study, the expression of *c-Fos* mRNA was higher in the ipsilateral side when compared to the contralateral side of the dorsal horn of the spinal cord of all animals as expected. Similarly, Okine et al from our group has reported higher levels of *c-Fos* mRNA expression in the ipsilateral side of the dorsal horn of spinal cord when compared to the contralateral side, one-hour post-formalin injection in rats that received intra-ACC administration of 100% DMSO vehicle (Okine et al., 2016). Modulation of the activity of dorsal horn neurons is a key outcome/endpoint following activation of the descending pain pathway (Bojovic et al., 2015; Gao and Ji, 2009). The fact that the behavioural effects of the drugs administered are not accompanied by drug-induced effects on *c-Fos* expression in the dorsal horn may suggest that the behavioural effects of pharmacological manipulation of TRPV1 in the DLPAG are not mediated by modulation of the descending pain pathway. However, drug-induced effects on formalin-induced nociceptive behaviour were transient (most effects were at the start and peak of the second phase) and all the effects had subsided by the end of formalin trial (i.e. at 60 mins). The 60 min duration of the formalin trial was chosen on the basis of studies demonstrating the duration of action of 5'-IRTX and capsaicin after intracerebroventricular injection in various pain models/tests (de Novellis et al., 2012; Maione et al., 2007, 2006; McGaraughty et al., 2003; Starowicz et al., 2007) and also because *c-Fos* mRNA levels in the dorsal horn of spinal cord have been shown to peak ~60 mins post-formalin (Abbadie et al., 1994, 1992; Abbadie and Besson, 1994; Gogas et al., 1991; Leah et al., 1996; Presley et al., 1990). Measurement of *c-Fos* mRNA has is

own limitations; in this study, I did not investigate the specific neuronal subtypes in which the *c-Fos* mRNA was expressed (e.g. using double labelling *in situ* hybridization) and the possibility that *c-Fos* may have been significantly altered in specific neuronal subtypes. *c-Fos* mRNA was assessed at a single time point which might not have been optimal to capture effects of the drugs given that their peak effects on behaviour were at the start and peak of the second phase of formalin-evoked nociceptive behaviour.

In conclusion, intra-DLPAG administration of either the TRPV1 agonist capsaicin, or the TRPV1 antagonist 5'-IRTX, significantly increased formalin-evoked nociceptive behaviour in SD rats, but not in WKY rats. The effects of capsaicin were likely due to TRPV1 desensitisation, given their similarity to the effects of 5'-IRTX. Taken together with the data presented in Chapter 2, the results suggest that lower expression and/or differential responsivity of TRPV1 in the DLPAG of WKY vs SD rats is associated with increased inflammatory pain behaviour and may underpin the hyperalgesic phenotype of WKY rats.

Chapter 4: The effects of pharmacological modulation of TRPV1 in the ventrolateral periaqueductal grey on formalin-evoked nociceptive behaviour in Wistar-Kyoto and Sprague-Dawley rats

4.1. Introduction

The VLPAG is known to play a key role in descending modulation of pain and several studies have demonstrated that electrical stimulation of this region inhibits ascending pain transmission (Cannon et al., 1982; De Luca-Vinhas et al., 2006). Unlike the LPAG, the VLPAG is activated upon noxious stimulation of deep tissue (Bandler et al., 2000; D P Finn et al., 2003; Keay et al., 1997; Keay and Bandler, 2001, 1993). As previously discussed, the PAG is functionally divided into columns which participate in active and passive coping strategies (Bandler and Keay, 1996; Keay and Bandler, 2001). Stimulation of the VLPAG results in passive coping which includes quiescence, hypotension and bradycardia (Keay and Bandler, 2001). Several cortical regions such as medial, ventrolateral, ventral, dorsolateral orbital, and dorsal and posterior insular agranular cortices project to the VLPAG (Floyd et al., 2000). Thalamic terminations from the PAG were mainly observed from the VLPAG and LPAG (Krout and Loewy, 2000). The VLPAG is the only subdivision which sends direct projections to the dorsal raphe nucleus (Kalén et al., 1985). Projections from the CeA to the VLPAG are known to play a role in modulation of pain (Rizvi et al., 1991) and are involved in anxiety/fear-related behaviour (Fox et al., 2015; Rizvi et al., 1991). For example, stimulation of the CeA increased latency to respond in the tail-flick test of thermal nociception, an effect abolished after administration of lidocaine intra-VLPAG (De Oliveira et al., 2001). Furthermore, intra-CeA administration of NMDA increased vocalization during the tail-flick test in a dose-dependent manner, an effect associated with increased *c-Fos* expression in VLPAG (Spuz et al., 2014), suggesting that the CeA and VLPAG are interconnected and play an important role in pain and aversion.

Direct administration of the TRPV1 agonist capsaicin into the VLPAG has been shown to induce antinociception, while administration of the TRPV1 antagonist 5'-IRTX evoked hyperalgesia, in the tail-flick and Hargreaves tests in rats (Maione et al., 2006; Starowicz et al., 2007). These effects were associated with an increase (capsaicin) or decrease (5'-IRTX) in glutamate release, respectively, in the RVM (Starowicz et al., 2007). Furthermore, the VLPAG plays a crucial role in opioid-induced hyperalgesia (Giesler and Liebeskind, 1976; Jensen and Yaksh, 1986; Mehalick et al., 2013; Sharpe

et al., 1974). Intra-VLPAG administration of morphine produced antinociception in the tail flick test by inhibiting GABAergic neurons, leading to the disinhibition of VLPAG output neurons projecting to the RVM and modulating spinal pain transmission (Vaughan et al., 1999, 1997). Evidence for interactions between TRPV1 and the opioid system comes from the work of Maione et al. who showed that the TRPV1 agonist capsaicin, when coadministered with the mu-opioid receptor agonist [D-Ala(2),N-Me-Phe(4),Gly(5)-ol]enkephalin into the VLPAG, stimulated glutamate release in the RVM and inhibited ON-cell neuronal activity in the RVM which in turn resulted in antinociception in a test of thermal nociception (Maione et al., 2009). These effects were antagonized by the TRPV1 antagonist 5'-IRTX and the opioid receptor antagonist naloxone administered intra-VLPAG, thus suggesting the existence of a TRPV1- μ -opioid receptor interaction in the VLPAG-RVM circuitry (Maione et al., 2009).

Studies from our laboratory by Jennings et al have investigated the role of the CB₁ receptor in the VLPAG on formalin-evoked nociceptive behaviour in WKY versus SD rats. The work demonstrated that pharmacological modulation of the CB₁ receptor in the VLPAG had no effect on formalin-evoked nociceptive behaviour in SD rats and WKY rats (Jennings, 2015). These data suggest that the CB₁ receptor does not play a role in modulation of hyperalgesia associated with negative affective state at the level of VLPAG. No study to date has evaluated the role of TRPV1 in the VLPAG in hyperalgesia associated with negative affect. The results presented in chapter 2 indicated that formalin administration increased TRPV1 mRNA expression in the VLPAG of SD rats but not in WKY rats. SD rats receiving an intra-plantar injection of saline had similar levels of TRPV1 mRNA in the VLPAG when compared to their respective WKY counterparts. These data suggest that SD and WKY rats have differential expression of TRPV1 expression in the presence of an inflammatory pain state. Therefore, this chapter examined the hypothesis that such a change in expression and/or functionality of TRPV1 in the VLPAG may play a role in the hyperalgesia exhibited by WKY rats, compared with SD counterparts.

Aims of the studies described in this chapter:

- To investigate the effects of pharmacological modulation of TRPV1 in the VLPAG on formalin-evoked nociceptive behaviour in WKY rats, compared with SD rats.
- To assess the effects of pharmacological modulation of TRPV1 in the VLPAG on levels of neurotransmitters and endocannabinoids/*N*-acylethanolamines in the RVM and dorsal horn of the spinal cord of formalin-injected WKY and SD rats.
- To investigate whether pharmacological modulation of TRPV1 in the VLPAG differentially alters *c-Fos* mRNA expression in the dorsal horn of the spinal cord of formalin-injected WKY and SD rats.

4.2 Materials and Methods

4.2.1 Animals

Details on animal supply and husbandry for the study described in this Chapter were identical to those described in Chapter 3, section 3.2.1 .

4.2.2 Experimental design

In the study described in this chapter, I investigated the effects of pharmacological modulation of TRPV1 in the VLPAG on formalin-evoked nociceptive behaviour in SD and WKY rats. The experimental design was identical to that described in Chapter 2 except that the VLPAG was targeted rather than the DLPAG. Male SD and WKY rats were implanted bilaterally under isoflurane anaesthesia with stainless steel guide cannulae targeting the VLPAG. On the test day, animals received bilateral intra-VLPAG injections of either vehicle (100% DMSO), the TRPV1 agonist capsaicin (CAP; 6nmoles/0.2 μ L), the TRPV1 antagonist 5'-IRTX (0.5nmoles/0.2 μ L) or co-administration of capsaicin and 5'-IRTX, and were placed in the formalin test arena for 10 minutes before intra-plantar formalin injection (2.5%, 50 μ l) under brief isoflurane anaesthesia. Rats were then returned to the formalin test arena and behaviour was recorded for a period of 60 minutes. Rats were killed by decapitation following behavioural testing. A 0.2 μ L quantity of 1% fast green dye was microinjected via the guide cannulae, and brains were rapidly removed, snap-frozen on dry ice, and stored at -80°C until injection site verification.

	Drug treatment			
	Vehicle	Capsaicin	5'-IRTX	Capsaicin+5'-IRTX
SD rats	5	7	6	6
WKY rats	5	5	6	5

Table 4.1: n numbers per group that received intra-VLPAG injections bilaterally post cannula verification.

4.2.3 Cannulae implantation

After acclimatization to the animal unit for 4-8 days after delivery, the rats were placed under brief isoflurane anaesthesia (2-3% in O₂, 0.5L/min), stainless steel guide cannulae (9mm length, Plastics One Inc., USA) were stereotactically implanted bilaterally 1mm above the VLPAG. The following coordinates were used for implanting cannulae; SD rats: AP = ((difference from Bregma to lambda) X 0.91mm) from Bregma, ML = ±1.9mm at an angle of 10°, DV = 5.3mm from the meningeal dura matter; WKY rats: AP = ((difference from Bregma to lambda) X 0.91mm) from Bregma, ML = ± 1.8mm at an angle of 10°, DV = 5.5mm from the meningeal dura matter according to the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998). The 9mm cannulae were permanently fixed to the skull using stainless steel screws and carboxylate cement (Durelon TM, USA). A stylet made from stainless steel tubing (9mm, 31 G) (Plastics One Inc., USA) was inserted into the guide cannulae to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (5mg/kg, s.c., Rimadyl, Pfizer, UK), was administered before the surgery to manage postoperative analgesia. To prevent postoperative infection, rats received a single daily dose of the antimicrobial agent enrofloxacin (2.5mg/kg, s.c., Baytril, Bayer plc, UK) on the day of surgery and for the 3 subsequent days. Following cannulae implantation, the rats were housed singly and allowed at least 5 days recovery prior to experimentation. During this recovery period, the rats were handled, cannulae checked, and their body weight and general health monitored once daily.

4.2.4 Drug preparation

Refer to Section 3.2.4 in Chapter 3.

4.2.5 Microinjections

Refer to Section 3.2.5 in Chapter 3.

4.2.6 Formalin test

Refer to Section 3.2.6 in Chapter 3.

4.2.7 Tissue harvesting

4.2.7.1 Brain removal and dissection

Refer to Section 2.2.4.1 in Chapter 2.

4.2.7.2 Spinal cord removal and dissection

Refer to Section 2.2.4.2 in Chapter 2.

4.2.8 Histological verification of microinjection sites

The sites of intracerebral microinjection were determined before data analysis and only those rats that had cannulae correctly positioned in the VLPAG were included in the final analysis. Brain sections with fast-green dye mark were collected on a cryostat (30µm thickness), mounted on gelatinised glass slides, and counterstained with cresyl violet as described in Chapter 3, Section 3.2.8 to locate the precise position of microinjection sites under light microscopy.

4.2.9 Tissue grinding

Refer to section 3.2.9. in Chapter 3.

4.2.10 Quantitative real-time PCR

Refer to Section 3.2.10. in Chapter 3.

4.2.11 Assay of Neurotransmitters and Endocannabinoid/N-acylethanolamine levels in the spinal cord using LC-MS/MS

Refer to Section 3.2.11 in Chapter 3.

4.2.12 Data analysis

Refer to chapter 3 Section 3.2.12. Data analysis.

4.3 Results

4.3.1 Histological verification of injector site location

Following sectioning of PAG, injection sites were verified under a light microscope, and only those injections placed within the borders of both the left and right VLPAG in SD and WKY rats were included in the final analyses. 24/34 and 21/33 of the injections were placed within the borders of both the right and left VLPAG in SD and WKY rats respectively (Fig. 4.1), with the remaining 10/34 and 12/33 having one or both cannulae positioned in the DLPAG or LPAG, or outside the PAG in the deep white layer of the superior colliculus. (Refer to Table 4.1 for final n numbers).

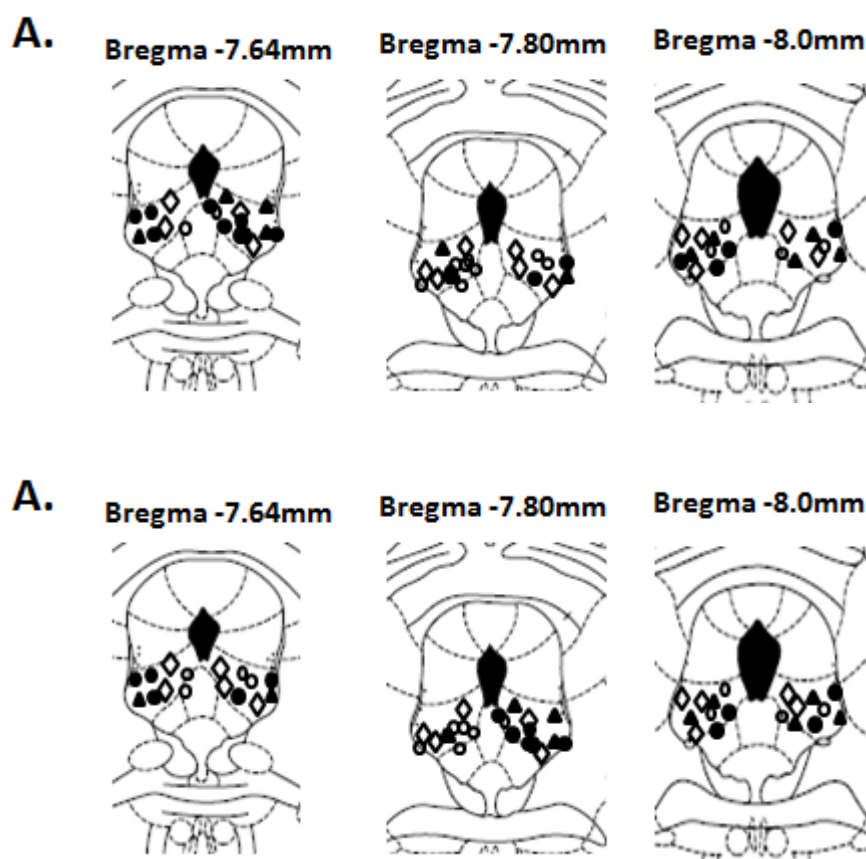


Figure 4.1: Schematic representation of vehicle (○) or capsaicin (▲) or 5'-IRTX (◇) or combination of capsaicin and 5'-IRTX (●) injections into the VLPAG of (A) SD and (B) WKY rats.

4.3.2 Effects of bilateral intra-VLPAG administration of a TRPV1 agonist, antagonist, or their combination, on general exploratory behaviours of SD and WKY rats during the pre-formalin trial

Intra-VLPAG administration of capsaicin or 5'-IRTX or the combination of both (Table 4.2) had no significant effect on locomotor activity, grooming or defecation in either SD or WKY rats when compared to vehicle-treated controls during the 10 min pre-formalin trial.

Group	Distance moved(cm)	Grooming(s)	Rearing(s)	Defecation (pellet number)
SD-Vehicle	1860.0±57.2	13.4±3.4	65.0±4.6	0 (0-1)
SD-CAP	1785.4±143.2	21.9±5.6	58.0±10.4	0.5 (0-1)
SD-5'-IRTX	1767.6±78.7	15.2±4.5	69.6±4.2	0 (0-1)
SD-CAP+5'-IRTX	1782.7±84.9	23.2±5.7	63.8±12.3	0 (0-1)
WKY-Vehicle	1693.6±30.4	10.4±4.2	50.3±8.2	0
WKY-CAP	1736.3±106.1	28.0±8.3	75.2±6.9	0 (0-1)
WKY-5'-IRTX	1787.3±74.3	26.1±8.6	45.6±8.3	0 (0-1)
WKY-CAP+5'-IRTX	1902.6±149.1	16.6±4.1	65.6±6.7	0 (0-2)

Table 4.2: Effects of intra-VLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,38}=0.076$, $P=0.784$); drug treatment ($F_{3,38}=0.27$, $P=0.847$) and strain X drug treatment interaction ($F_{1,38}=0.741$, $P=0.534$); Grooming: Two-way ANOVA effects of strain ($F_{1,38}=0.186$, $P=0.669$); drug treatment ($F_{3,38}=1.544$, $P=0.219$) and strain X drug treatment interaction ($F_{1,38}=0.898$, $P=0.451$); Rearing: Two-way ANOVA effects of strain ($F_{1,38}=0.701$, $P=0.408$); drug treatment ($F_{3,38}=0.637$, $P=0.596$) and strain X drug treatment interaction ($F_{1,38}=2.432$, $P=0.08$); Defecation: Kruskal Wallis analysis test by rank ($X^2=1.329$, $P=0.988$). Data are expressed as mean ± SEM for parametric data and median (interquartile range) for nonparametric data (n = 5 - 8 rats per group)

4.3.3 Effects of intra-VLPAG administration of capsaicin, 5'-IRTX or their combination on formalin-evoked nociceptive behaviour and on general exploratory/locomotor behaviours during the formalin test in SD and WKY rats.

Two-way repeated measures ANOVA revealed a significant effect of time ($P < 0.001$) strain X time interaction ($P < 0.001$) time X drug treatment interaction ($P < 0.05$) and time X strain X drug treatment interaction ($P < 0.05$) on formalin-evoked nociceptive behaviour. WKY rats that received intra-VLPAG administration of vehicle exhibited significantly greater nociceptive behaviour, compared with SD counterparts (Fig 4.2a WKY-Vehicle vs SD-Vehicle, $P < 0.05$, $P < 0.01$), confirming the hyperalgesic phenotype in the WKY strain. In SD rats, intra-VLPAG administration of capsaicin (Fig 4.2b SD-CAP vs SD-Vehicle, $P < 0.05$) or 5'-IRTX (Fig 4.2b SD-5'-IRTX vs SD-Vehicle, $P < 0.05$) or the combination of both drugs (Fig 4.2b SD-CAP+5'-IRTX vs SD-Vehicle, $P < 0.05$) significantly reduced formalin-evoked nociceptive behaviour intermittently in the second phase of the formalin trial, compared with vehicle-treated SD rats. These effects were not observed in WKY rats except for 5'-IRTX which had reduced nociceptive behaviour at one-time bin in WKY rats (Fig 4.2c WKY-5'-IRTX vs WKY-Vehicle, $P < 0.05$).

Two-way ANOVA revealed significant strain X drug treatment ($P < 0.01$) interaction on grooming behaviour during the post-formalin trial period. Intra-VLPAG administration of capsaicin reduced grooming behaviour in WKY rats, compared to SD counterparts (Table 4.3 $P < 0.01$, SD-Capsaicin vs WKY-Capsaicin). No effect of strain or drug treatment was observed on rearing, grooming or defecation over this period (Table 4.3). There were no significant differences between the groups for change in hind paw diameter (Fig 4.3).

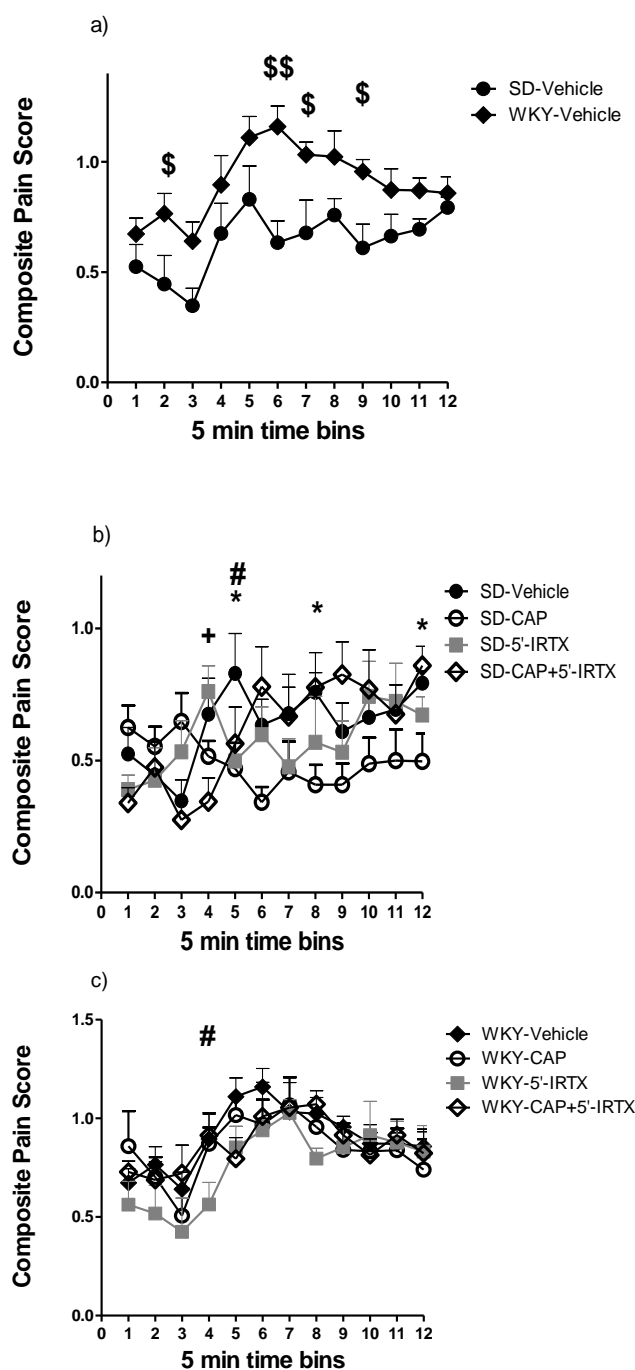


Figure 4.2 (a) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving intra-VLPAG administration of vehicle. (b) Intra-VLPAG administration of the TRPV1 agonist capsaicin or the TRPV1 antagonist 5'-IRTX reduced formalin-evoked nociceptive behaviour in SD rats. (c) Intra-VLPAG administration of 5'-IRTX, but not capsaicin, transiently reduced formalin-evoked nociceptive behaviour in WKY rats. Two-way repeated measures ANOVA, time: $F_{11,396} = 9.294, P < 0.001$; effects of time \times strain: $F_{11,396} = 3.012, P < 0.001$; time \times drug treatment : $F_{33,396} = 1.650, P < 0.05$; and time \times strain \times drug treatment interaction: $F_{33,396} = 1.580, P < 0.05$) followed by Fisher's LSD post hoc test (Fig 3a $^{\$}P < 0.05$, $^{$$}P < 0.01$, SD-Vehicle vs WKY-Vehicle; Fig 3b $^*P < 0.05$, SD-CAP vs SD-Vehicle; $^{\#}P < 0.05$, SD-5'-IRTX vs SD-Vehicle; $^{+}P < 0.05$, SD-CAP+5'-IRTX vs SD-Vehicle; Fig 3c $^{\#}P < 0.05$, WKY-5'-IRTX vs WKY-Vehicle). Data are expressed as mean \pm SEM (n = 5 - 8 rats per group).

Group	Distance moved(cm)	Grooming(s)	Rearing(s)	Defecation (pellet number)
SD-Vehicle	14779.9±1868	5.8±3.4	3.2±1.2	0.5 (0-3)
SD-Capsaicin	15315.3±2440.1	12.2±2.9	15.7±7.2	2 (1-2)
SD-5'-I-RTX	12221.5±2347.9	2.7±1.6	6.6±3.6	1 (1-2)
SD- CAP+5'-IRTX	11907.6±491.2	2.5±1.6	24.9±19.5	2.5 (0-2)
WKY-Vehicle	7208.1±1118.5	1.0±1.0	1.4±0.9	0.5 (0-1)
WKY-Capsaicin	6431.6±452.11	0**	1.0±0.1	0 (0-1)
WKY-5'-I-RTX	16497±9073.5	8.3±4.8	4.3±2.4	0.5 (0-2)
WKY- CAP+5'-IRTX	6347.6±599.9	0	0	2 (0-2)

Table 4.3: Effects of intra-VLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation during the 60 min formalin trial in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,45}=2.148$, $P = 0.151$); drug treatment ($F_{1,45}=0.558$, $P = 0.646$) and strain X drug treatment interaction ($F_{1,45}= 1.051$, $P = 0.381$); Grooming: Two-way ANOVA effects of strain ($F_{1,45}=2.927$, $P = 0.095$); drug treatment ($F_{3,45}= 1.175$, $P = 0.332$) and strain X drug treatment interaction ($F_{1,45}=3.452$, $P < 0.01$); Rearing: Two-way ANOVA effects of strain ($F_{1,45}=3.927$, $P = 0.055$); drug treatment ($F_{3,45}=0.556$, $P = 0.647$) and strain X drug treatment interaction ($F_{1,45}= 0.983$, $P = 0.411$) followed by Fisher's LSD post hoc test ($^{**}P < 0.01$, SD-Capsaicin) Defecation: Kruskal Wallis variance of analysis by rank ($X^2 = 5.457$, $P = 0.604$). Data are expressed as mean \pm SEM for parametric data and median (interquartile range) for nonparametric data (n = 5 - 8 rats per group).

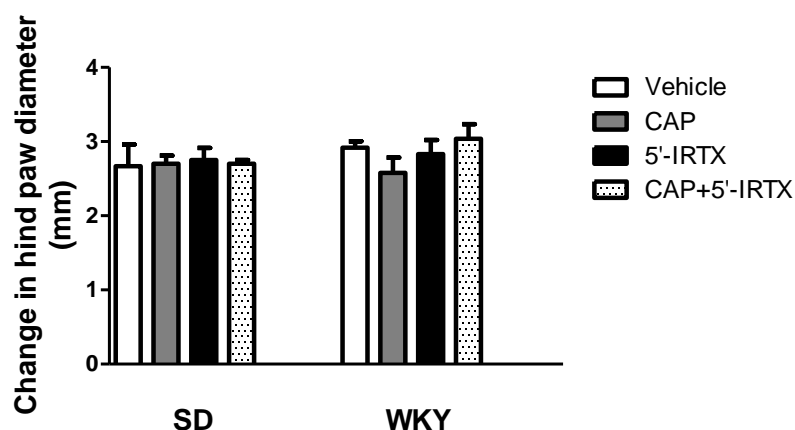


Figure 4.3: Difference in paw diameter (mm) pre- and post-formalin injection. Two-way ANOVA effects of strain ($F_{1,35} = 1.228$, $P = 0.275$); drug treatment ($F_{3,35} = 0.569$, $P = 0.639$) and strain X drug treatment interaction ($F_{1,35} = 0.635$, $P = 0.597$) followed by Fisher's LSD post hoc test. Data are means \pm S.E.M, $n=5-8$. (SD: Sprague-Dawley, WKY: Wistar-Kyoto).

4.3.4 Effects of intra-VLPAG administration of vehicle, capsaicin, 5'-IRTX and the combination of capsaicin and 5'-IRTX on levels of neurotransmitters and endocannabinoids/N-acylethanolamines in the RVM of formalin-injected SD and WKY rats.

Intra-VLPAG administration of capsaicin, 5'-IRTX or their combination had no effect on the neurotransmitter levels (GABA, Glutamate and Serotonin) or the levels endocannabinoids/N-acylethanolamines (Table 4.4) in RVM tissue of SD rats. Intra-VLPAG administration of capsaicin, 5'-IRTX and the combination of capsaicin and 5'-IRTX had no effect on neurotransmitters or on AEA and OEA in the RVM of formalin-injected WKY rats. Two-way ANOVA revealed significant effect of drug treatment ($P < 0.01$) on 2-AG levels in the RVM after pharmacological modulation of TRPV1 in the VLPAG. Further pairwise comparisons using post hoc revealed intra-VLPAG administration of 5'-IRTX alone did significantly increase the levels of 2-AG in WKY rats (Table 4.4 $P < 0.05$, $P < 0.01$ WKY-5'-IRTX vs WKY-vehicle). Two-way ANOVA revealed significant strain effect on serotonin ($P < 0.01$) and PEA ($P < 0.05$) levels respectively, in the RVM after pharmacological modulation of TRPV1 in the VLPAG. WKY rats had significantly higher levels of PEA and lower levels of Serotonin in WKY rats when compared to SD counterparts (Table 4.4). Intra-VLPAG administration of 5'-IRTX alone or in combination with capsaicin did significantly increase the levels of PEA in WKY rats (Table 4.4 $P < 0.05$, WKY-5'-IRTX vs WKY-vehicle, WKY-CAP+5'-IRTX vs WKY-vehicle).

Group	GABA	Glutamate	Serotonin	AEA	2-AG	PEA	OEA
	μmol/g	μmol/g	nmol/g	picomol/g	nmol/g	nmol/g	nmol/g
	tissue	tissue	tissue	tissue	tissue	tissue	tissue
	weight	weight	weight	weight	weight	weight	weight
SD-Vehicle	6.4±1.5	24.9±6.3	2.4±0.6	302.1±83.7	60.7±8.2	1.9±0.3	1.3±0.3
SD-Capsaicin	5.7±0.8	21.3±3.5	2.5±0.5	173.2±28.8	94.6±27.8	1.5±0.3	1.3±0.2
SD-5'-IRTX	5.8±1.4	20.2±6.5	2.0±0.4	219.6±35.2	89.1±20.3	1.7±0.3	1.6±0.3
SD- CAP+5'-IRTX	6.5±0.8	20.3±1.7	2.6±0.4	226.7±46.1	73.7±11.5	1.7±0.2	1.4±0.2
WKY-Vehicle	3.7±0.4	14.3±1.5	1.5±0.2	221.2±68.0	57.2±9.8	1.5±0.1	1.1±0.1
WKY-Capsaicin	5.3±0.7	19.1±3.8	1.4±0.2	269.5±68.4	67.9±11.8	2.1±0.2	1.7±0.3
WKY-5'-IRTX	6.1±1.3	21.3±4.7	1.2±0.4	311.6±77.2	136.9±38.1 ^{**}	2.7±0.3 [*]	1.9±0.3
WKY- CAP+5'-IRTX	4.9±0.6	19.1±2.9	1.6±0.5	439.2±149.5	73.4±16.7	2.8±0.7 ^{*^}	2.1±0.7

Table 4.4: Effects of intra-VLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on neurotransmitter levels and endocannabinoid levels in the RVM post formalin trial in SD and WKY rats. GABA: Two-way ANOVA effects of strain ($F_{1,44}=2.547$, $P = 0.12$); drug treatment ($F_{3,44}=0.959$, $P = 0.423$) and strain X drug treatment interaction ($F_{3,44}= 0.249$, $P = 0.862$); Glutamate: Two-way ANOVA effects of strain ($F_{1,45}=1.262$, $P = 0.269$); drug treatment ($F_{3,45}= 0.125$, $P = 0.945$) and strain X drug treatment interaction ($F_{1,45}=0.825$, $P = 0.49$); Serotonin: Two-way ANOVA effects of strain ($F_{1,46}=7.78$, $P < 0.01$); drug treatment ($F_{3,46}= 0.773$, $P = 0.518$) and strain X drug treatment interaction ($F_{1,46}=0.292$, $P = 0.831$); AEA: Two-way ANOVA effects of strain ($F_{1,45}= 2.938$, $P = 0.096$); drug treatment ($F_{3,45}=0.874$, $P = 0.465$) and strain X drug treatment interaction ($F_{3,45}=0.832$, $P = 0.486$); 2-AG: Two-way ANOVA effects of strain ($F_{1,46}= 0.167$, $P = 0.686$); drug treatment ($F_{3,46}=3.239$, $P<0.05$); and strain X drug treatment interaction ($F_{3,46}=1.02$, $P = 0.396$); PEA: Two-way ANOVA effects of strain ($F_{1,46}= 5.818$, $P < 0.05$); drug treatment ($F_{3,46}=2.31$, $P = 0.094$) and strain X drug treatment interaction ($F_{3,46}=0.837$, $P =0.483$); OEA: Two-way ANOVA effects of strain ($F_{1,46}=2.031$, $P = 0.164$); drug treatment ($F_{3,46}= 1.527$, $P = 0.226$) and strain X drug treatment interaction ($F_{3,46}=0.352$, $P = 0.788$); followed by Fisher's LSD post hoc test; ($*P<0.05$ $**P<0.01$ vs WKY-vehicle) ($^{\wedge}P<0.05$ vs SD-CAP+5'-IRTX); Data are expressed as mean \pm SEM (n = 5 - 7 rats per group).

4.3.5 Effects of intra-VLPAG administration of vehicle, capsaicin, 5'-IRTX and the combination of capsaicin and 5'-IRTX on levels of neurotransmitters and endocannabinoids/N-acylethanolamines in the dorsal horn of the spinal cord (ipsilateral and contralateral sides) of formalin-injected SD and WKY rats.

Intra-VLPAG administration of vehicle, capsaicin, 5'-IRTX and combination of capsaicin and 5'-IRTX had no effect on the levels of GABA, glutamate, AEA, 2-AG, PEA and OEA in either SD or WKY rats on either side of dorsal horn of spinal cord (Table 4.5). Three-way ANOVA revealed significant differences between the ipsilateral and contralateral sides of dorsal horn of spinal cord for the neurotransmitters (glutamate and serotonin) and endocannabinoids/N-acylethanolamines (AEA, 2-AG, OEA and PEA) (Table 4.5 $P < 0.05$, $P < 0.01$, $P < 0.001$ vs respective ipsilateral counterparts). Three-way ANOVA revealed significant strain effect ($P < 0.01$) on the glutamate and PEA levels in the dorsal horn of spinal cord. Three-way ANOVA revealed significant side X strain interaction effect ($P < 0.01$) on the glutamate and GABA levels in the dorsal horn of spinal cord. Three-way ANOVA revealed significant strain X drug treatment interaction effect ($P < 0.05$) on serotonin levels in the dorsal horn of spinal cord. Further pairwise comparisons using post hoc analysis revealed glutamate and PEA were higher in WKY rats on the ipsilateral side when compared to SD counterparts (Table 4.5). In SD rats, on the ipsilateral side of the spinal cord, intra-VLPAG administration of capsaicin decreased the levels of serotonin, compared with vehicle-treated controls (Table 4.5 $P < 0.05$, SD-Capsaicin vs SD-Vehicle). On the ipsilateral side of the spinal cord, vehicle-treated WKY rats had higher levels of GABA when compared to their SD counterparts (Table 4.5 $P < 0.05$, WKY-vehicle vs SD-vehicle). On the ipsilateral side of the spinal cord, WKY rats that received intra-VLPAG capsaicin had higher levels of glutamate when compared to their SD counterparts (Table 4.5 $P < 0.05$, WKY-Capsaicin vs SD-Capsaicin). On the ipsilateral side of the spinal cord, WKY rats that received intra-VLPAG capsaicin in combination with 5'-IRTX had higher levels of glutamate when compared to their SD counterparts (Table 4.5 $P < 0.05$, WKY-Capsaicin+5'-IRTX vs SD-Capsaicin+5'-IRTX). On the ipsilateral side of spinal cord, WKY rats that received intra-VLPAG administration of vehicle or the combination of capsaicin and 5'-IRTX- had higher levels of PEA when compared to their SD counterparts (Table 4.5 $P < 0.05$, WKY-vehicle vs SD-vehicle; $P < 0.05$, WKY-Capsaicin+5'-IRTX vs SD-Capsaicin+5'-IRTX).

Group	GABA µmol/g tissue weight	Glutamate µmol/g tissue weight	Serotonin nmol/g tissue weight	AEA picomol/g tissue weight	2-AG nmol/g tissue weight	PEA nmol/g tissue weight	OEA nmol/g tissue weight
Contralateral side of DHSC							
SD-Vehicle	4.6±0.4	14.1±1.5	6.4±1.8	90.2±12.9	83.0±15.5	1.4±0.2	1.6±0.2
SD-Capsaicin	4.6±0.4	14.5±1.9	4.0±0.6	67.5±7.6	92.6±19.7	1.3±0.1	1.6±0.1
SD-5'-I-RTX	5.3±0.6	17.2±2.1	5.1±0.5	72.2±7.8	99.5±8.3	1.6±0.4	1.9±0.4
SD- CAP+5'-I-RTX	5.3±1.0	17.0±3.6	5.9±1.0	83.5±18.2	105.2±11.6	1.6±0.2	2.0±0.3
WKY-Vehicle	5.0±0.5	16.2±2.0	4.2±1.1	91.3±11.4	120.5±11.3 ^s	1.5±0.2	1.9±0.2
WKY-Capsaicin	4.3±0.2	16.0±0.8	4.5±0.8	81.8±10.8	118.6±16.6	1.9±0.2 ^{&}	1.9±0.1
WKY-5'-I-RTX	4.1±1.2	10.4±0.5	4.0±0.7	52.3±9.6	105.5±11.3	1.3±0.1	1.3±0.2
WKY- CAP+5'-I-RTX	3.6±0.5	11.7±1.0	3.6±0.8	68.4±9.9	89.4±4.1	1.6±0.2	1.7±0.1
Ipsilateral side of DHSC							
SD-Vehicle	4.7±0.5	38.4±5.4 ^{ff}	10.9±2.0	34.5±6.6 ^{fff}	29.1±3.9 ^{fff}	1.6±0.3	1.2±0.2
SD-Capsaicin	4.9±0.9	33.8±3.7 ^{ff}	4.7±0.6 [*]	37.7±8.2 ^f	31.6±5.4 ^{fff}	1.6±0.1	1.1±0.1
SD-5'-I-RTX	4.2±1.2	30.2±8.8 ^{ff}	6.9±1.9	37.2±12.6	24.3±4.8 ^{fff}	1.7±0.5	1.4±0.4
SD- CAP+5'-I-RTX	4.0±0.4	30.2±3.9	7.4±2.1	16.9±2.4 ^{fff}	21.7±2.5 ^{fff}	1.4±0.1	1.0±0.0 ^{ff}
WKY-Vehicle	6.5±0.4 ^s	53.0±2.9 ^{fff}	6.4±1.0	51.0±8.7 ^{ff}	35.2±5.6 ^{fff}	2.3±0.3 st	1.4±0.2
WKY-Capsaicin	5.3±1.0	49.6±10.0 ^{&fff}	8.1±1.2	33.2±7.5 ^{ff}	24.2±3.0 ^{fff}	2.1±0.4	1.3±0.2 ^f
WKY-5'-I-RTX	5.9±1.2	52.1±10.4 ^{fff}	6.5±1.5	40.4±7.8	28.5±2.8 ^{fff}	2.0±0.2	1.2±0.2
WKY- CAP+5'-I-RTX	5.5±1.0	52.0±7.4 ^{^fff}	4.3±0.9	33.9±6.7 ^f	31.7±7.1 ^{fff}	2.1±0.2 [^]	1.4±0.1

Table 4.5: Effects of intra-VLPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on neurotransmitter levels and endocannabinoids/N-acylethanolamines in the ipsilateral side of dorsal horn of spinal cord in SD and WKY rats. GABA: Three-way ANOVA effects of side ($F_{1,82}=1.161$, $P = 0.285$); strain ($F_{1,82}=1.449$, $P = 0.233$); drug treatment ($F_{3,82}=0.226$, $P = 0.878$); side X strain ($F_{1,82}= 9.365$, $P < 0.01$); side X drug treatment ($F_{3,82}= 0.067$, $P = 0.977$) strain X drug treatment ($F_{3,82}= 0.789$, $P = 0.505$); side X strain X drug treatment ($F_{3,82}= 0.677$, $P = 0.569$). Glutamate: Three-way ANOVA effects of side ($F_{1,82}=112.803$, $P < 0.001$); strain ($F_{1,82}=8.271$, $P < 0.01$); drug treatment ($F_{3,82}=0.237$, $P = 0.87$); side X strain ($F_{1,82}= 11.382$, $P < 0.01$); side X drug treatment ($F_{3,82}= 0.145$, $P = 0.823$) strain X drug treatment ($F_{3,82}= 0.083$, $P = 0.969$); side X strain X drug treatment ($F_{3,82}= 0.381$, $P = 0.767$). Serotonin: Three-way ANOVA effects of side ($F_{1,82}=10.253$, $P < 0.01$); strain ($F_{1,82}=2.832$, $P =0.097$); drug treatment ($F_{3,82}=0.789$, $P = 0.504$); side X strain ($F_{1,82}= 0.083$, $P = 0.775$); side X drug treatment ($F_{3,82}= 0.277$, $P = 0.842$) strain X drug treatment ($F_{3,82}= 2.872$, $P < 0.05$); side X strain X drug treatment ($F_{3,82}= 0.432$, $P = 0.731$). AEA: Three-way ANOVA effects of side ($F_{1,82}=65.329$, $P < 0.001$); strain ($F_{1,82}=0.252$, $P =0.617$); drug treatment ($F_{3,82}=1.609$, $P = 0.195$); side X strain ($F_{1,82}= 0.829$, $P = 0.366$); side X drug treatment ($F_{3,82}= 1.251$, $P = 0.298$) strain X drug treatment ($F_{3,82}= 0.451$, $P = 0.717$); side X strain X drug treatment ($F_{3,82}= 1.2$, $P= 0.316$). 2-AG: Three-way ANOVA effects of side ($F_{1,82}=205.525$, $P < 0.001$); strain ($F_{1,82}=2.936$, $P =0.091$); drug treatment ($F_{3,82}=0.277$, $P = 0.842$); side X strain ($F_{1,82}= 1.629$, $P = 0.206$); side X drug treatment ($F_{3,82}= 0.122$, $P = 0.947$) strain X drug treatment ($F_{3,82}= 0.451$, $P = 0.341$); side X strain X drug treatment ($F_{3,82}= 1.755$, $P= 0.164$). PEA: Three-way ANOVA effects of side ($F_{1,83}=5.186$, $P < 0.05$); strain ($F_{1,83}=9.244$, $P <0.01$); drug treatment ($F_{3,83}=0.064$, $P = 0.979$); side X strain ($F_{1,83}= 3.373$, $P = 0.071$); side X drug treatment ($F_{3,83}= 0.126$, $P = 0.944$) strain X drug treatment ($F_{3,83}= 0.859$, $P = 0.467$); side X strain X drug treatment ($F_{3,83}= 0.803$, $P= 0.496$). OEA: Three-way ANOVA effects of side ($F_{1,83}=24.8$, $P < 0.001$); strain ($F_{1,83}=0.436$, $P =0.511$); drug treatment ($F_{3,83}=0.037$, $P = 0.99$); side X strain ($F_{1,83}= 0.63$, $P = 0.43$); side X drug treatment ($F_{3,83}= 0.336$, $P = 0.799$) strain X drug treatment ($F_{3,83}= 1.947$, $P = 0.13$); side X strain X drug treatment ($F_{3,83}= 0.609$, $P= 0.612$), Followed by Fisher's LSD post hoc test; (* $P < 0.05$, vs SD-Vehicle) ($^{\text{t}}P < 0.05$, $^{\text{tt}}P < 0.01$, $^{\text{ttt}}P < 0.001$ vs respective contralateral counterparts) ($^{\&}P < 0.05$, $^{\&\&}P < 0.01$, WKY-Capsaicin vs SD-Capsaicin) ($^{\text{s}}P < 0.05$, WKY-vehicle vs SD-vehicle) ($^{\text{^}}P < 0.05$, $^{\text{^^}}P < 0.01$, WKY-Capsaicin+5'-IRTX vs SD- Capsaicin+5'-IRTX). Data are expressed as mean \pm SEM (n = 5 - 7 rats per group).

4.3.6 Effects of intra-VLPAG administration of capsaicin, 5'-IRTX, or their combination on *c-Fos* mRNA expression in the dorsal horn of the spinal cord in SD rats and WKY rats.

There was no difference in *c-Fos* mRNA expression between the ipsilateral and contralateral sides of SD and WKY-Vehicle treated rats (Fig 4.4). Administration of capsaicin or 5'-IRTX, alone or in combination, had no effect on *c-Fos* mRNA expression in the dorsal horn of the spinal cord of SD and WKY rats, compared to their vehicle-treated counterparts irrespective of the side (Fig 4.4).

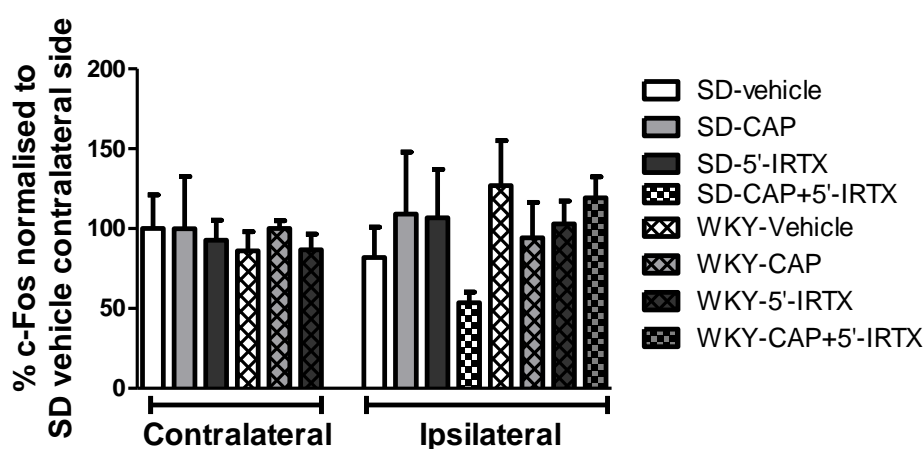


Figure 4.4: *c-Fos* mRNA expression on the ipsilateral and contralateral sides of dorsal horn of the spinal cord after intra-DLPAG administration of capsaicin or 5'-IRTX or vehicle or combination of both capsaicin and 5'-IRTX in SD and WKY rats. Three-way ANOVA: effects of side ($F_{1,68} = 0.467$, $P = 0.497$); strain ($F_{1,68} = 0.886$, $P = 0.351$); drug treatment ($F_{3,68} = 0.263$, $P = 0.851$); side X strain interaction ($F_{1,68} = 0.314$, $P = 0.578$); side X drug treatment interaction ($F_{2,68} = 0.087$, $P = 0.917$); strain X drug treatment interaction ($F_{3,68} = 0.862$, $P = 0.467$); side X strain X drug treatment interaction ($F_{2,68} = 0.772$, $P = 0.467$). Data are means \pm S.E.M, $n=5-7$. (SD: Sprague-Dawley, WKY: Wistar-Kyoto).

4.4 Discussion:

The data presented in this chapter demonstrate that TRPV1 in the VLPAG column regulates formalin-evoked nociceptive behaviour differentially in SD rats versus WKY counterparts. In SD rats, intra-VLPAG administration of either capsaicin or 5'-IRTX reduced formalin-evoked nociceptive behaviour intermittently when compared to vehicle-treated SD rats. Co-administration of capsaicin and 5'-IRTX also decreased formalin-evoked nociceptive behaviour at the early second phase of the trial in SD rats. In contrast, pharmacological manipulation of TRPV1 with intra-VLPAG administration of either capsaicin alone or in combination with 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in WKY rats. In WKY rats, intra-VLPAG 5'-IRTX decreased formalin-evoked nociceptive behaviour at the early second phase of the formalin trial, when compared to vehicle-treated counterparts. Intra-VLPAG administration of capsaicin, 5'-IRTX or their combination had no effects on levels of neurotransmitters or endocannabinoid/N-acylethanolamines in the dorsal horn of the spinal cord in WKY rats, however some differences were observed between discrete SD and WKY groups. No significant effects of drug treatment were seen on neurotransmitter levels in the RVM of WKY rats. Intra-VLPAG administration of 5'-IRTX alone or in combination with capsaicin increased PEA levels in the RVM when compared to vehicle-treated WKY rats, an effect not seen in SD rats. In WKY rats, intra-VLPAG administration of 5'-IRTX increased 2-AG levels in the RVM, an effect not seen in SD rats. No effects of the side, strain or drug treatment on *c-Fos* mRNA expression in the spinal cord were observed.

Direct microinjection of the TRPV1 agonist capsaicin into the VLPAG reduced formalin-evoked nociceptive behaviour at discrete time periods in SD rats. In contrast, WKY rats were non-responsive to intra-VLPAG capsaicin. Formalin-evoked nociceptive behaviour in SD rats was also associated with higher TRPV1 expression in the VLPAG (chapter 2). Accordingly, capsaicin-induced desensitisation of TRPV1 in the VLPAG had an antinociceptive effect in SD rats. In contrast, WKY rats were non-responsive to intra-VLPAG capsaicin, possibly because formalin-treated WKY rats had lower TRPV1 expression in the VLPAG compared with SD counterparts. Such an effect on TRPV1 expression in WKY rats may constitute a compensatory change to counter the hyperalgesic response to formalin exhibited by this strain. Both strains exhibited modest and transient antinociceptive effects following intra-VLPAG administration of

5'-IRTX alone, or in combination with capsaicin. Studies from de Novellis and colleagues have demonstrated that intra-VLPAG administration of AA-5HT, a FAAH inhibitor and TRPV1 antagonist, was antinociceptive in the rat formalin test, an effect associated with reduced ON cell and OFF cell activity in the RVM (de Novellis et al., 2008). Furthermore, intra-VLPAG AA-5-HT increased the firing of neurons in the locus coeruleus (LC) and intrathecal phentolamine (antagonist for 5HTA3 receptors) or ketanserin (antagonist of 5-HT receptors) prevented the analgesic effect of AA-5-HT. Thus the authors propose that a PAG - LC - spinal cord pathway may be implicated in the effects of intra-VLPAG AA-5HT (de Novellis et al., 2008). Moreover, intra-VLPAG AA-5-HT prevented the changes in ON and OFF cell firing activity induced by intra-plantar formalin injection, and it prevented the formalin-induced increase in LC noradrenergic cell activity (de Novellis et al., 2008). In a more recent study, Liao et al reported that capsaicin, when injected into the VLPAG, increased paw withdrawal latency in the hot plate test in Wistar rats (Liao et al., 2011), similar to the capsaicin-induced reduction in formalin-evoked nociceptive behaviour in SD rats observed in the present study. They suggested that capsaicin activates TRPV1 on glutamatergic neurons in the VLPAG, resulting in mGluR5-mediated production of 2-AG which in turn results in retrograde disinhibition of GABAergic neurons with consequent activation of the descending inhibitory pain pathway and antinociception (Liao et al., 2011). Intra-VLPAG microinjection of 5'-IRTX alone reduced the latency to nociceptive response (pronociceptive effect) in the plantar test and this effect of 5'-IRTX was abolished when co-administered with capsaicin (Starowicz et al., 2007).

Intra-VLPAG administration of capsaicin, 5'-IRTX, or capsaicin in combination with 5'-IRTX had no effect on rearing, grooming or distance moved during the pre-formalin trial when compared to vehicle- treated controls in either SD or WKY rats. Furthermore, the drugs had no effect on grooming, rearing, or distance moved during the formalin trial in either strain when compared to the vehicle-treated controls. However, grooming behaviour was lower in capsaicin-treated WKY rats during the formalin-trial period when compared to SD counterparts. Previous studies have demonstrated that exploratory/locomotor behaviours such as rearing and grooming are lower in WKY rats when compared to SD rats (Burke et al., 2010; Paré, 1994). Paw oedema developed similarly in both strains and all drug treatment groups indicating that formalin injections were performed consistently across all the groups and the effects of intra-VLPAG drug

treatment on formalin-evoked nociceptive behaviour are unrelated to an effect on hind paw oedema.

The present study also examined the effect of pharmacological modulation of TRPV1 in the VLPAG on tissue levels of neurotransmitters and endocannabinoids/N-acylethanolamines in the RVM. Intra-VLPAG administration of 5'-IRTX alone increased the levels of 2-AG and PEA in the RVM of WKY rats. Irrespective of drug treatment, there were significant differences between the ipsilateral and contralateral sides of the dorsal horn of the spinal cord for the neurotransmitters (Glutamate, and Serotonin) and endocannabinoids/N-acylethanolamines (AEA, 2-AG, OEA and PEA). Administration of capsaicin alone decreased the levels of PEA only in WKY rats when compared to vehicle-treated counterparts on the ipsilateral side of dorsal horn of spinal cord. Administration of capsaicin in combination with 5'-IRTX increased the levels of PEA only in WKY rats when compared to vehicle-treated counterparts on the ipsilateral side of dorsal horn of spinal cord. Previous studies from our laboratory have shown that PEA levels in the RVM are low in formalin-treated WKY rats when compared to SD rats (Olango, 2012). Thus, it is possible that pharmacological modulation of TRPV1 counteracts the hyperalgesic response in WKY rats by modulating the PEA levels which lead to antinociception (Calignano et al., 1998; Conti et al., 2002; Helyes et al., 2003). Starowicz et al have reported that intra-VLPAG capsaicin-evoked a robust release of glutamate in RVM microdialysates (Starowicz et al., 2007). 5'-IRTX, at a dose inactive *per se*, blocked the effect of capsaicin and inhibited glutamate release. 5'-IRTX at a higher dose (0.5nmol/rat) decreased the glutamate levels significantly. Antinociception and hyperalgesia induced by capsaicin and 5'-IRTX in the tail flick test correlated with enhanced or reduced activity, respectively, of RVM OFF cells (Starowicz et al., 2007). These data suggest that VLPAG neurons respond to TRPV1 stimulation by releasing glutamate in the RVM, thereby activating OFF cells and producing analgesia. In the present study, intra-VLPAG injections of capsaicin or 5-IRTX, alone or in combination, had no effect on the GABA and glutamate levels in the RVM of SD or WKY rats exposed to the formalin test. The discrepancy might be due to different nociceptive behaviour tests (Starowicz et al. used tail-flick test and formalin test in the current study). However, it should also be noted that in the present study neurotransmitters and endocannabinoids were measured at a single time point i.e. 60 mins post formalin injection. Furthermore, each study employed different methods of analysis (microdialysis in Starowicz et al and LC-MS/MS on punch-dissected tissue levels in the

present study), and so we cannot differentiate between extracellular and intracellular levels of the GABA and glutamate in these samples when compared to microdialysis which assesses extracellular levels only. These methodological differences may explain the discrepancies between the neurochemical results reported herein and those previously published in the literature.

The current study also examined the expression of the neuronal activity marker *c-Fos* in the dorsal horn of the spinal cord. The data demonstrated that there was no difference in *c-Fos* mRNA expression on the ipsilateral side of the spinal cord when compared to the contralateral side of vehicle-injected SD or WKY rats. The discrepancy between these data and those reported in chapter 3 where *c-Fos* expression was higher on the ipsilateral side compared with the contralateral side might be due to factors such as the consistency of the dissection procedure between the two studies, or possible effects of intra-VLPAG DMSO on the descending pain pathway versus intra-DLPAG DMSO (Chapter 3). There were no effects of strain or intra-VLPAG administration of capsaicin, 5'-IRTX or their combination on *c-Fos* expression in the dorsal horn of the spinal cord, however the lack of effect of formalin injection on *c-Fos* expression on the ipsilateral side makes it very difficult to draw firm conclusions with respect to strain or drug treatment. It is possible that analysis of c-Fos protein (e.g. by immunohistochemistry) rather than mRNA may have been a more robust approach to reveal effects of formalin injection, strain and/or drug treatment.

In conclusion, the data presented in this chapter indicate that intra-VLPAG administration of capsaicin or 5'-IRTX reduces nociceptive behaviour in a moderate and transient manner in SD rats, and similar effects were seen with 5'-IRTX in WKY rats. The effects of capsaicin were likely due to TRPV1 desensitisation, given their similarity to the effects of 5'-IRTX, in SD rats. In Chapter 2, formalin-treated WKY rats had lower TRPV1 expression in the VLPAG compared with SD counterparts. Taken together with the data presented here, changes in TRPV1 expression in WKY rats may constitute a compensatory change to counter the hyperalgesic response to formalin exhibited by this strain.

Chapter 5: The effects of pharmacological modulation of TRPV1 in the lateral periaqueductal grey on formalin-evoked nociceptive behaviour in Wistar-Kyoto and Sprague-Dawley rats

5.1 Introduction:

Pain disorders pose a huge health and socioeconomic problem across the world. The ability of an organism to experience pain is essential to prevent tissue damage from noxious stimuli and is conducive to survival. Under normal circumstances, there is a physiological balance between inhibition and facilitation of pain to facilitate conscious perception of pain when needed. Hyperalgesia is a heightened pain response to normally painful stimuli and can be exacerbated by negative affective state, including anxiety and depression (Asmundson and Katz, 2009; Atkinson et al., 1991). As demonstrated in the previous chapters 2, 3 and 4, the WKY rat model of negative affective state also displays a hyperalgesic phenotype and represents a useful model with which to study the role of TRPV1 in the PAG in hyperalgesia associated with negative affect.

The LPAG receives input from the primary motor areas of the cortex (controlling hind limbs and forelimbs) (Newman et al., 1989), spinal cord and the spinal trigeminal nucleus (Keay et al., 1997; Keay and Bandler, 1992; Menétrey et al., 1980; Menétrey and De Pommery, 1991; Mouton et al., 2001, 1997; Mouton and Holstege, 1998; Vanderhorst et al., 1996; Wiberg et al., 1987, 1986, Yeziarski, 1991, 1988; Yeziarski and Broton, 1991; Yeziarski and Mendez, 1991). In turn, neurons project from the LPAG to the rostral and caudal regions of the ventromedial and ventrolateral medulla (Holstege, 1991; Holstege and Kuypers, 1982; Illing and Graybiel, 1986). Given these incoming and outgoing projections of the LPAG, it is not surprising that studies have demonstrated that the LPAG plays an important role in modulating nociception. LPAG activation has been shown to lead to non-opioid mediated analgesia (Bandler et al., 2000). Finn et al have reported an increase in expression of the marker of neuronal activity, c-Fos, in the LPAG after intraplantar formalin injection in male SD rats (D P Finn et al., 2003). Aside from its role in the modulation of nociception, activation of the LPAG also induces fight or flight defensive behaviours (Bandler et al., 1985; Bandler and Depaulis, 1988). For example, LPAG stimulation is known to elicit defecation, flight response and an increase in sympathetic nervous system activity

leading to tachycardia and hypertension (Bandler et al., 1991; Schenberg et al., 2005). Recently Linnman et al. have replicated the findings from previous studies (Bandler and Depaulis, 1988; Keay et al., 1997) demonstrating that cutaneous noxious stimulation is associated with activation of the LPAG (Linnman et al., 2012). Furthermore, retrograde neuronal tracing experiments combined with immunohistochemistry demonstrated that a number of LPAG neurons send direct projections to the nucleus ambiguus (NA) in the medulla, a major origin of parasympathetic preganglionic neurons to the heart and that these neurons are activated by white noise sound exposure (Koba et al., 2016). White noise sound exposure at 90 dB induced freezing behaviour and elicited bradycardia in conscious rats (Yoshimoto et al., 2010). Based on these findings, it is proposed that the LPAG/VLPAG-NA monosynaptic pathway transmits fear-driven central signals, which elicit bradycardia through parasympathetic outflow (Koba et al., 2016). Recently, optogenetic approaches have been used to study the role of basic synaptic properties of specific neural circuits in physiology and behaviour (Britt et al., 2012; Han, 2012). Channelrhodopsin-2 (ChR2), an algal protein from *Chlamydomonas reinhardtii*, is the most common light-sensitive protein used (Britt et al., 2012; Han, 2012). Assareh et al. used channel rhodopsin (ChR2) stimulation to probe defensive behaviour controlled by the LPAG; suprathreshold LPAG stimulation evoked escape-flight defence that was replaced by freezing at stimulation offset, hence providing more evidence that the LPAG is involved in mediating defence responses (Assareh et al., 2016).

Studies from Jennings et al. in our laboratory have focused on the role of the endocannabinoid system in the LPAG in formalin-evoked nociceptive behaviour in WKY versus SD rats (Jennings, 2015). In SD rats, intra-LPAG administration of ACEA (CB₁ receptor agonist) produced a CB₁ receptor-mediated reduction in formalin-evoked nociceptive behaviour that was associated with an increase and decrease in c-Fos expression in the RVM and dorsal horn respectively (Jennings, 2015). However, these effects were not observed in WKY rats receiving intra-LPAG administration of ACEA. These results suggest that CB₁ receptor activation in the LPAG of SD rats elicits an anti-nociceptive effect by activating the descending inhibitory pain pathway, while CB₁ receptors in the LPAG of WKY rats are hyporesponsive to the pharmacological challenge which fails to engage the descending inhibitory pain pathway. To date, however, no studies have investigated the role of TRPV1 in the LPAG on pain responding in WKY versus SD rats.

The results presented in chapter 2 demonstrated that TRPV1 (mRNA and protein) is expressed in all the columns of the PAG. Formalin administration had no effect on TRPV1 mRNA expression in the LPAG of SD or WKY rats. However, formalin-treated WKY rats had higher levels of TRPV1 mRNA in the LPAG when compared to formalin-treated SD rats. Based on previous pharmacological data presented in chapters 3 (DLPAG) and 4 (VLPAG), herein we completed our investigation of the role of TRPV1 in the 3 different PAG columns by testing the hypothesis that pharmacological modulation of TRPV1 in the LPAG differentially affects formalin-evoked nociceptive behaviour in WKY versus SD rats.

Aim of this chapter

- To investigate the effects of pharmacological modulation of TRPV1 in the LPAG on formalin-evoked nociceptive behaviour in WKY rats compared to SD rats.

5.2 Materials and Methods

5.2.1. Animals

Details on animal supply and husbandry for the study described in this Chapter 5 were identical to those described in Chapter 3, section 3.2.1.

5.2.2 Experimental design

In the study described in this chapter, I investigated the effects of pharmacological modulation of TRPV1 in the LPAG on formalin-evoked nociceptive behaviour in WKY rats and SD rats. Male SD and WKY rats were implanted bilaterally under isoflurane anaesthesia with stainless steel guide cannulae targeting the LPAG. On the test day, animals received bilateral intra-LPAG injections of either vehicle (100% DMSO), the TRPV1 agonist capsaicin (CAP; 6nmoles/0.2 μ L), the TRPV1 antagonist 5'-IRTX (0.5nmoles/0.2 μ L) or co-administration of capsaicin and 5'-IRTX, and were placed in the formalin test arena for 10 minutes before intra-plantar formalin injection (2.5%, 50 μ l) under brief isoflurane anaesthesia. Rats were then returned to the formalin test arena and behaviour was recorded for a period of 60 minutes. Rats were killed by decapitation following behavioural testing. A 0.2 μ L quantity of 1% fast green dye was microinjected via the guide cannula, and brains were rapidly removed, snap-frozen on dry ice, and stored at -80°C until injection site verification.

	Drug treatment			
	Vehicle	Capsaicin	5'-IRTX	Capsaicin+5'-IRTX
SD rats	9	12	10	11
WKY rats	8	9	12	10

Table 5.1: n numbers per group that received intra-LPAG injections bilaterally post-histological verification.

5.2.3 Cannulae implantation

After acclimatization for 4-8 days after delivery, the rats were placed under isoflurane anaesthesia (2-3% in O₂, 0.5L/min), stainless steel guide cannulae (9mm length, Plastics One Inc., USA) were stereotactically implanted bilaterally 1mm above the LPAG. The following coordinates were used for implanting cannulae in SD rats: AP = ((difference from Bregma to lambda) X 0.91mm) from Bregma, ML = ±1.9mm at an angle of 10°, DV = 5.0mm from the meningeal dura matter; WKY rats: AP = ((difference from Bregma to lambda) X 0.91mm) from Bregma, ML = ± 1.8mm at an angle of 10°, DV = 5.2mm from the meningeal dura matter according to the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998). The 9mm cannulae were permanently fixed to the skull using stainless steel screws and carboxylate cement (Durelon™, USA). A stylet made from stainless steel tubing (9mm, 31 G) (Plastics One Inc., USA) was inserted into the guide cannulae to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (5mg/kg, s.c., Rimadyl, Pfizer, UK), was administered before the surgery to manage postoperative analgesia. To prevent postoperative infection, rats received a single daily dose of the antimicrobial agent enrofloxacin (2.5mg/kg, s.c., Baytril, Bayer plc, UK) on the day of surgery and a subsequent 3 days. Following cannulae implantation, the rats were housed singly and allowed at least 5 days recovery prior to experimentation. During this recovery period, the rats were handled, cannulae checked, and their body weight and general health monitored once daily.

5.2.4 Drug preparation

Refer to Section 3.2.4 in Chapter 3.

5.2.5. Microinjection

Refer to Section 3.2.5 in Chapter 3.

5.2.6 Formalin Test

Refer to Section 3.2.6 in Chapter 3.

5.2.7 Tissue harvesting

5.2.7.1. Brain removal and dissection

Refer to chapter 2 Section 2.2.4.1. Brain removal.

5.2.7.2 Spinal cord removal and dissection

Refer to chapter 2 Section 2.2.4.2. Spinal cord removal.

5.2.8. Histological verification of microinjection sites

The sites of intracerebral microinjection were determined before data analysis and only those rats that had cannulae correctly positioned in both the right and left LPAG were included in the final analyses. Brain sections with fast-green dye mark were collected on a cryostat (30µm thickness), mounted on gelatinised glass slides, and counterstained with cresyl violet, mounted and coverslipped as described previously (Chapter 3, Section 3.2.8) to locate the precise position of microinjection sites under light microscopy.

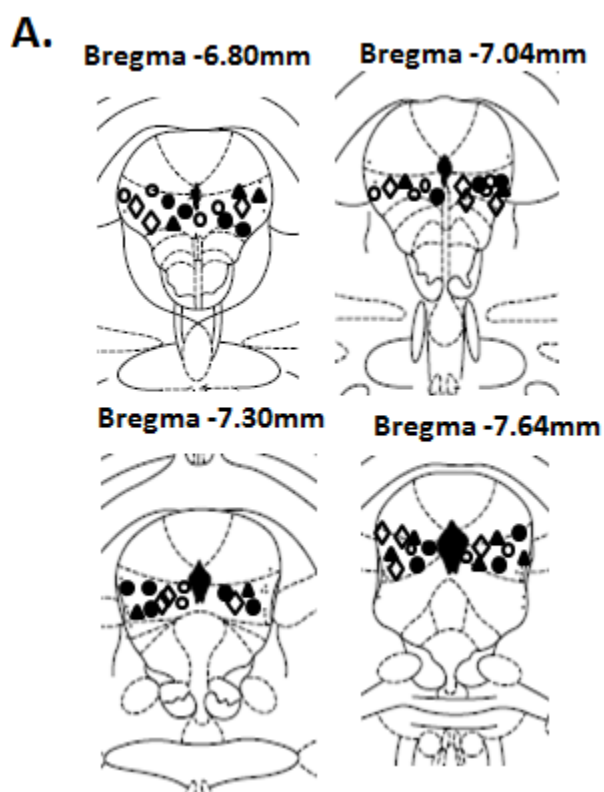
5.2.9. Data analysis

Refer to chapter 3 Section 3.2.12. Data analysis.

5.3 Results

5.3.1 Histological verification of injector site location

Following sectioning of the PAG, injection sites were verified under a light microscope, and only those injections placed within the borders of both the left and right LPAG in SD and WKY rats were included in the final analyses. 42/63 and 39/61 of the injections were placed within the borders of both the right and left LPAG in SD and WKY rats, respectively (Fig. 5.1), with the remaining 21/63 and 22/61 having one or both cannulae positioned in the dorsolateral/ventrolateral, or outside the PAG in the deep white layer of the superior colliculus. (Refer to Table 5.1 for final n numbers).



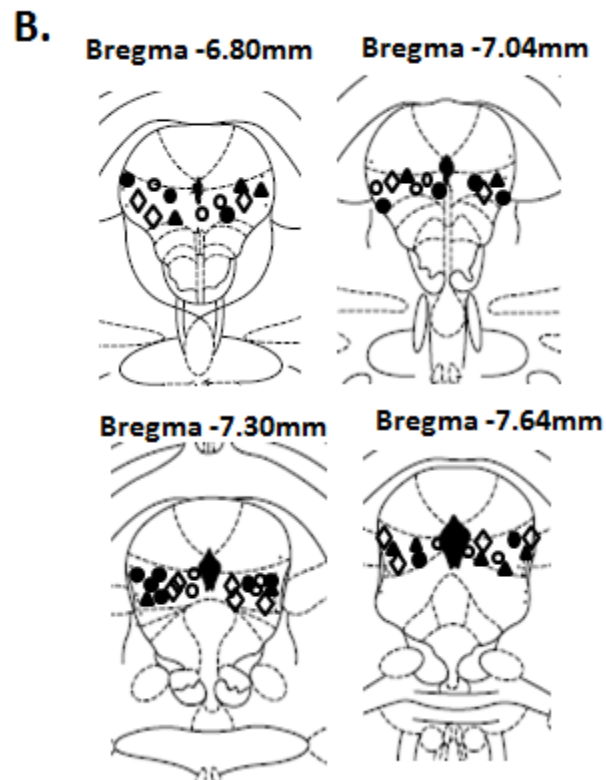


Figure 5.1 Schematic representation of vehicle (○) or capsaicin (▲) or 5'-IRTX (◇) or the combination of capsaicin and 5'-IRTX (●) injections into the LPAG of (A) SD and (B) WKY rats.

5.3.2 Effects of bilateral intra-LPAG administration of a TRPV1 receptor agonist, antagonist, or their combination, on general exploratory/locomotor behaviours during the pre-formalin trial

Intra-LPAG administration of capsaicin and 5'-IRTX, alone or in combination had no significant effect on locomotor activity, grooming or defecation in either SD or WKY rats when compared to vehicle-treated controls during the 10 min pre-formalin trial (Table 5.1). Two-way ANOVA revealed significant strain effect ($P < 0.01$) on rearing behaviour during the pre-formalin trial. Further post hoc analysis revealed, vehicle- and 5'-IRTX-treated WKY rats exhibited lower rearing activity compared with SD counterparts (Table 5.1: $P < 0.05$ WKY-Vehicle vs SD-Vehicle, $P < 0.05$ WKY-5'-IRTX vs SD-5'-IRTX).

Group	Distance moved(cm)	Grooming(s)	Rearing(s)	Defecation (pellet number)
SD-Vehicle	1749.9±86.4	20.1±7.4	61.04±10.1	0.5 (0-1)
SD-CAP	1687.4±75.1	31.6±4.9	55.7±6.0	0.5 (0-1)
SD-5'-IRTX	1487.6±71.3	21.1±3.5	72.0±10.6	0 (0-1)
SD-CAP+5'-IRTX	1602.1±87.7	17.7±4.4	58.7±7.0	0
WKY-Vehicle	1471.4±102.4	38.2±7.4	28.1±5.6 [§]	1 (1-2)
WKY-CAP	1597.3±82.4	25.9±6.6	52.4±4.9	1 (0-1.5)
WKY-5'-IRTX	1554.3±96.0	27.3±6.0	44.5±7.8 [#]	0 (0-1)
WKY-CAP+5'-IRTX	1551.5±144.0	19.8±4.9	52.6±11.3	1 (0-1)

Table 5.2: Effects of intra-LPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,79}=1.710$, $P = 0.195$); drug treatment ($F_{3,79}=0.639$, $P = 0.592$) and strain X drug treatment interaction ($F_{1,79}= 1.075$, $P = 0.364$); Grooming: Two-way ANOVA effects of strain ($F_{1,79}=1.668$, $P=0.200$); drug treatment ($F_{3,79}= 1.439$, $P = 0.279$) and strain X drug treatment interaction ($F_{1,79}=1.410$, $P = 0.246$); Rearing: Two-way ANOVA effects of strain ($F_{1,79}=8.341$, $P < 0.01$); drug treatment ($F_{3,79}=0.899$, $P = 0.446$) and strain X drug treatment interaction ($F_{1,79}= 1.494$, $P = 0.223$) followed by Fisher's LSD post hoc test ([§] $P < 0.05$, vs SD-Vehicle; [#] $P < 0.05$, vs SD-5'-IRTX);

Defecation: Kruskal Wallis analysis of variance by rank ($X^2 = 8.562$, $P = 0.286$). Data are expressed as mean \pm SEM for parametric data and median (interquartile range) for nonparametric data (n = 8 – 12 rats per group).

5.3.3 Effects of intra-LPAG administration of capsaicin, 5'-IRTX or the combination of both on formalin-evoked nociceptive behaviour in SD rats and WKY rats

Two-way repeated measures ANOVA revealed a significant effect of time ($P < 0.001$), time X strain interaction ($P < 0.001$), time X drug treatment interaction ($P < 0.01$) on formalin-evoked nociceptive behaviour. Further pairwise comparisons using post hoc analysis revealed that WKY rats that received intra-LPAG administration of vehicle exhibited higher nociceptive behaviour, compared with SD counterparts (Fig 5.2a WKY-Vehicle vs SD-Vehicle, $P < 0.05$), confirming the hyperalgesic phenotype in the WKY strain. In SD rats, intra-LPAG administration of 5'-IRTX significantly reduced formalin-evoked nociceptive behaviour intermittently from 5-10 min and from 20-25 min post-formalin, compared with vehicle-treated SD rats (Fig 5.2b SD-5'-IRTX vs SD-Vehicle, $P < 0.05$), an effect not observed in WKY rats (Fig 5.2c). Intra-LPAG administration of capsaicin alone, or in combination with 5'-IRTX, had no effect on formalin-evoked nociceptive behaviour in SD (Fig 5.2b) or WKY rats (Fig 5.2c) when compared to respective vehicle-treated controls.

Two-way ANOVA revealed a significant effect of strain ($P < 0.01$) on rearing and grooming behaviour during the post-formalin trial. Further pairwise comparisons using *Post hoc* analysis revealed intra-LPAG vehicle-treated WKY rats exhibited lower grooming behaviour when compared to their SD counterparts (Table 5.3 $P < 0.01$, WKY-Vehicle vs SD-Vehicle). In SD rats, intra-LPAG administration of capsaicin and 5'IRTX, alone or in combination, decreased grooming behaviour significantly when compared to vehicle-treated controls (Table 5.3 $P < 0.01$, vs SD-Vehicle). Capsaicin-treated WKY rats exhibited lower rearing activity when compared to SD counterparts (Table 5.3 $P < 0.05$, WKY-CAP vs SD-CAP).

There were no effects of strain or intra-LPAG administration of capsaicin and 5'-IRTX, alone or in combination, on the change in hind paw diameter post-formalin injection (Fig 5.3).

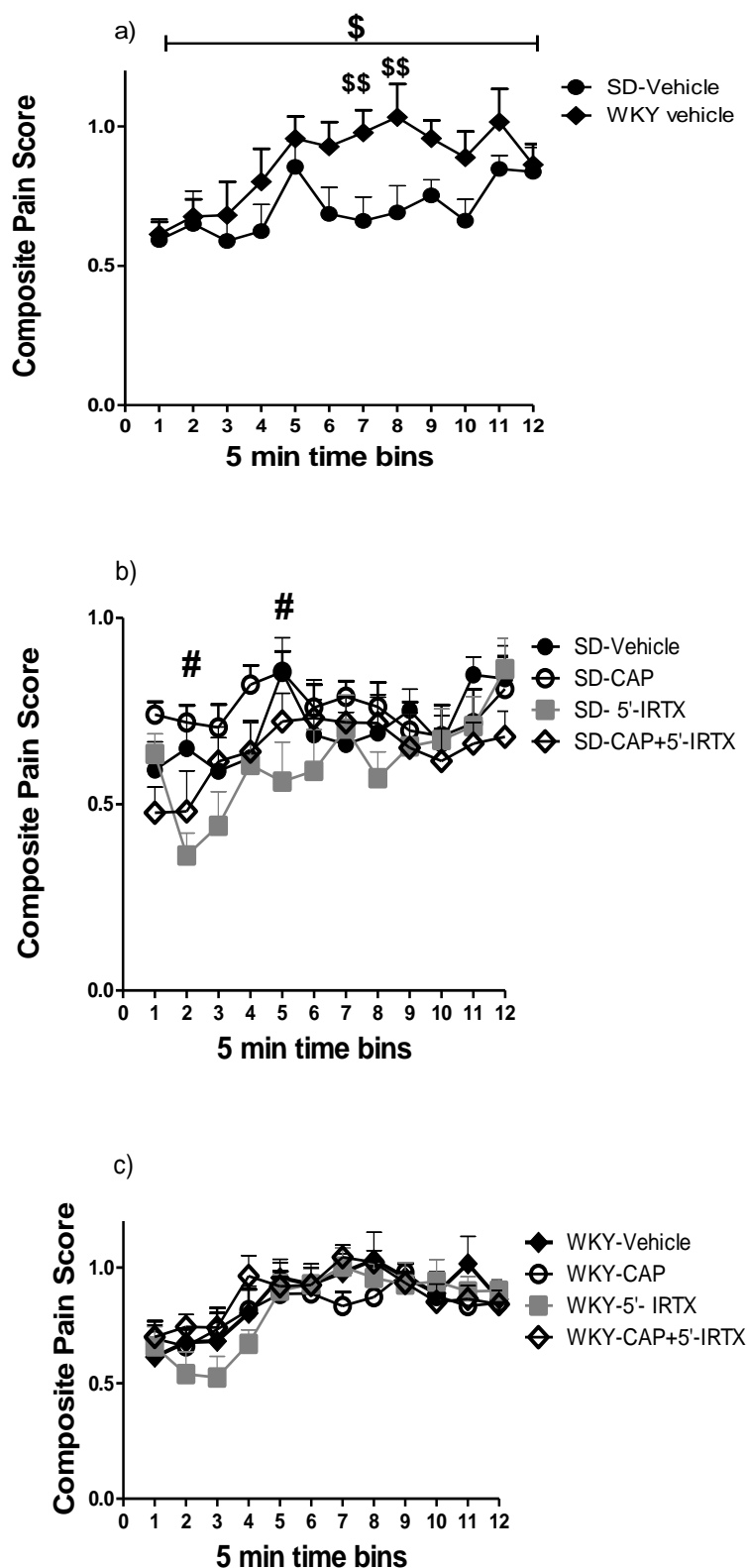


Figure 5.2: (a) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats receiving intra-LPAG administration of vehicle. Intra-LPAG administration of 5'-IRTX reduced formalin-evoked nociceptive behaviour in (b) SD rats, but not in (c) WKY rats. Two-way repeated measures ANOVA, time: $F_{11,781} = 15.463$, $P < 0.001$; time \times strain: $F_{11,781} = 2.492$, $P < 0.01$; time \times drug treatment: $F_{33,781} = 1.818$, $P < 0.01$; and time \times strain \times drug treatment interaction: $F_{33,781} = 0.724$, $P = 0.874$ followed by Fisher's LSD post hoc test (Fig 5a $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, SD-Vehicle vs WKY-Vehicle) (Fig 5b $^{\#}P < 0.05$, SD-5'-IRTX vs SD-Vehicle). Data are expressed as mean \pm SEM (n = 8 – 11 rats per group).

Group	Distance moved(cm)	Grooming(s)	Rearing(s)	Defecation (pellet number)
SD-Vehicle	8933.4±4317.9	13.8±4.3	19.5±10.1	1 (0-3)
SD-Capsaicin	10382.7±4719.1	5.5±2.3**	23.1±11.0	2 (0-3)
SD-5'-I-RTX	7617.8±1964.6	3.4±1.7**	12.8±4.4	0.5 (0-2)
SD- CAP+5'-IRTX	5239.7±282.6	3.4±1.4**	5.67±2.5	1 (0-2)
WKY-Vehicle	3599.9±796.3	3.4±1.3**	0.75±0.4	2 (0-3)
WKY-Capsaicin	8591.0±3797	4.5±1.7	0.48±0.3 [#]	1 (0-3)
WKY-5'-I-RTX	4886.5±1741.4	3.51±1.4	1.15±0.5	1 (0-2)
WKY- CAP+5'-IRTX	2953.2±293.2	1.86±0.74	1.22±0.4	1.5 (1-3)

Table 5.3: Effects of intra-LPAG microinjection of either vehicle, capsaicin, 5'-IRTX or the combination of capsaicin and 5'-IRTX on locomotor activity, grooming and defecation during the 60 min formalin trial in SD and WKY rats. Distance moved: Two-way ANOVA effects of strain ($F_{1,82}=1.993$, $P = 0.162$); drug treatment ($F_{1,82}=1.110$, $P = 0.351$) and strain X drug treatment interaction ($F_{1,82}= 0.124$, $P = 0.946$); Grooming: Two-way ANOVA effects of strain ($F_{1,82}=4.248$, $P < 0.05$); drug treatment ($F_{3,82}= 2.644$, $P = 0.055$) and strain X drug treatment interaction ($F_{1,82}=2.220$, $P = 0.492$); Rearing: Two-way ANOVA effects of strain ($F_{1,82}=10.209$, $P < 0.01$); drug treatment ($F_{3,82}=0.899$, $P = 0.565$) and strain X drug treatment interaction ($F_{1,82}= 0.811$, $P = 0.492$) followed by Fisher's LSD post hoc test ($**P < 0.01$, SD-Vehicle ; $^{\#}P < 0.05$, SD-CAP) Defecation: Kruskal Wallis variance of analysis by rank ($X^2 = 4.29$, $P = 0.746$). Data are expressed as mean \pm SEM for parametric data and median (interquartile range) for nonparametric data (n = 8 - 12 rats per group).

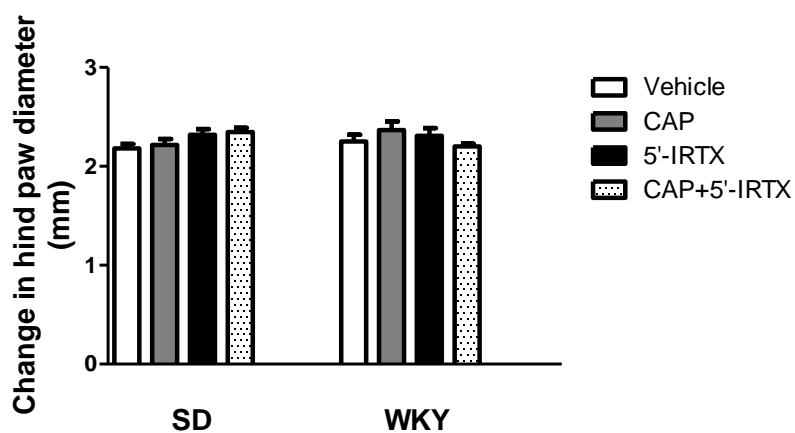


Figure 5.3: Difference in hind paw diameter (mm) pre- and post-formalin injection. Two-way ANOVA (effects of strain ($F_{1,35} = 0.132$, $P = 0.717$); drug treatment ($F_{3,35} = 0.868$, $P = 0.462$) and strain X drug treatment interaction ($F_{1,35} = 2.116$, $P = 0.105$). Data are means \pm S.E.M, $n=8-12$. (SD: Sprague-Dawley, WKY: Wistar-Kyoto).

5.4 Discussion:

WKY rats that received intra-LPAG administration of vehicle exhibited higher formalin-evoked nociceptive behaviour compared to SD counterparts, thus confirming the hyperalgesic phenotype of WKY rats. Intra-LPAG administration of the TRPV1 antagonist 5'-IRTX reduced the formalin-evoked nociceptive behaviour intermittently during the first 25 min of the formalin-trial period in SD rats, but not in WKY rats. Intra-LPAG administration of capsaicin alone, or in combination with 5'-IRTX, had no effect on formalin-evoked nociceptive behaviour in either strain. Grooming behaviour during the formalin trial was significantly lower in vehicle-treated WKY rats, compared to SD counterparts. Intra-LPAG administration of capsaicin and 5'-IRTX, alone or in combination significantly reduced grooming in SD rats but not WKY rats. WKY rats exhibited more rearing behaviour when compared to SD rats during the pre-formalin trial period.

In Chapter 2, TRPV1 mRNA expression in the LPAG of WKY rats was higher than that of SD rats. The formalin injection had no effect on TRPV1 mRNA or protein expression in the LPAG of either strain, suggesting that TRPV1 expression in the LPAG column is unaffected by a noxious inflammatory stimulus. While there was no change in TRPV1 expression associated with formalin-evoked pain, WKY rats exhibited higher TRPV1 expression in the LPAG compared with SD rats. Thus, the ability of 5'-IRTX to reduce formalin-evoked nociceptive behaviour in SD rats might relate to the lower expression of TRPV1 in the LPAG of SD rats. In comparison, WKY rats exhibit higher expression of TRPV1 in the LPAG and thus the dose of 5'-IRTX used herein might not be sufficiently high to bind and inhibit TRPV1 in the LPAG and produce an effect.

Administration of capsaicin into the LPAG had no effect on formalin-evoked nociceptive behaviour in either SD or WKY rats. To my knowledge, the present study is the first to investigate the effects of TRPV1 modulation in the LPAG on nociceptive behaviour and the data suggest that TRPV1 modulation in this PAG column has little (in SD rats) or no effect (in WKY rats) on formalin-evoked nociceptive behaviour. It is likely that other receptors might be playing a role in pain modulation at the level of the LPAG such as GPR55, CB₁ or P2X₃ receptors. Intra-PAG (LPAG/VLPAG) injection of lysophosphatidylinositol (LPI), a GPR55 agonist, reduced latencies in the hot plate test in SD rats. The effect of LPI was blocked by the GPR55 antagonist ML-193. These findings suggest that GPR55 in the LPAG/VLPAG is pronociceptive (Deliu et al.,

2015). Similarly, P2X3 receptor expression in the LPAG is decreased in parallel with a reduction in pain thresholds in the chronic constriction injury model of neuropathic pain in SD rats. Intra-LPAG administration of the P2X3 agonist α,β -methylene-ATP (α, β -meATP) attenuated the nociceptive behaviour in this model and administration of the antagonist A-317491 blocked these effects (Xiao et al., 2010). In SD rats, intra-LPAG administration of the CB₁ receptor agonist ACEA reduced formalin-evoked nociceptive behaviour in SD rats but had no effect in WKY rats (Jennings, 2015). It is possible therefore that the antinociceptive effects of intra-LPAG administration of 5'-IRTX observed in the present study are due to shunting of AEA towards an action at CB₁ receptors in the presence of TRPV1 blockade, with a consequent CB₁ receptor-mediated reduction in formalin-evoked nociceptive behaviour in SD rats, but not WKY rats. Further experimentation would be necessary to test this hypothesis.

WKY rats receiving intra-LPAG administration of vehicle or 5'-IRTX exhibited reduced rearing when compared to SD counterparts during the pre-formalin trial period. These data support previous reports of lower rearing activity in WKY rats when compared to SD rats (Burke et al., 2010; Paré, 1994). Intra-LPAG administration of any drug treatment had no effect on grooming, distance moved or defecation during the 10 mins pre-formalin trial. These data suggest that intra-LPAG drug treatment (capsaicin, 5'-IRTX alone or in combination with capsaicin) has no effect on general exploratory/locomotor behaviours during the pre-formalin trial in SD or WKY rats. The lower levels of grooming observed in vehicle-treated WKY rats compared to SD counterparts during the formalin trial might be due to the fact that WKY rats were hyperalgesic and expressing greater formalin-evoked nociceptive behaviour than SD rats during the trial. Concomitantly, intra-LPAG administration of capsaicin, 5'-IRTX, or their combination, had no effects on grooming in WKY rats but did reduce grooming in SD rats where 5'-IRTX also had a modest and transient antinociceptive effect. However, the results do also suggest that it is possible to dissociate grooming and formalin-evoked nociceptive behaviour because in SD rats intra-LPAG administration of capsaicin, or capsaicin + 5'-IRTX, reduced the former while having no effect on the latter. Intra-LPAG administration of capsaicin, 5'-IRTX, or their combination, had no effect on the distance moved, irrespective of the strain, compared to vehicle treatment during the post-formalin trial period, suggesting that drug treatment had no effect on locomotor activity in the presence of formalin-evoked nociceptive behaviour. There was a significant effect of strain on rearing activity, and examining the data it is clear that

WKY rats (irrespective of drug treatment) exhibit reduced rearing activity during the post-formalin trial similar to the effect observed in the pre-formalin trial. Differential effects of intra-LPAG administration of capsaicin, 5'-IRTX or their combination on general or locomotor behaviours in the pre- versus post-formalin trials (e.g. rearing and grooming) might be attributable to various factors including, the pharmacokinetics of the drugs, absence versus presence of formalin-evoked nociceptive tone, time of administration of the drugs, and strain effects. Formalin-evoked hind paw oedema developed similarly in both strains and all drug treatment groups, suggesting that formalin injections were performed consistently across all treatment groups and pharmacological modulation of TRPV1 in the LPAG had no effect on hind paw oedema in either strain.

Given the lack of effect of intra-LPAG administration of capsaicin, and very modest effects of 5'-IRTX at only two discrete 5 min time intervals in SD rats only during the early part of the formalin trial, we felt it was not possible to justify further post-mortem work to measure *c-Fos* expression in the dorsal horn or levels of neurotransmitters and endocannabinoids/N-acylethanolamines in the RVM as had been done for the studies described in chapters 3 and 4 where more robust drug effects and strain differences were observed.

In conclusion, the data presented in this chapter suggest that pharmacological modulation of TRPV1 in the LPAG has little effect on formalin-evoked nociceptive behaviour in SD rats (in contrast to the results for the DLPAG (chapter 3) and VLPAG (chapter 4)) and is unlikely to make a significant contribution to the hyperalgesic phenotype of WKY rats.

Chapter 6: General Discussion

The first aim of the work described in this thesis was to examine inflammatory pain behaviour in an animal model of negative affect, the WKY rat, and to determine if behavioural changes were accompanied by alterations in TRPV1 expression within the columns of the PAG. Following this, I investigated the effects of pharmacological manipulation of TRPV1 in the columns of the PAG of SD and WKY rats on inflammatory pain behaviour and associated activation of the descending inhibitory pain pathway. This chapter will consider the most significant behavioural and neurobiological findings described herein, and discuss how these contribute to increasing our understanding of pain exacerbation in negative affective states. It will also consider some limitations of the work described, and highlight some areas worthy of future investigation.

The major contributions of the work described in this thesis include:

- a) Characterization of anxiety-related and formalin-evoked nociceptive behaviour in the WKY rat, versus SD rats, and associated alterations in TRPV1 mRNA and protein levels in columns of the PAG, a region important in modulating both negative affective state and pain.
- b) Demonstration that in SD rats, intra-DLPAG administration of either the TRPV1 agonist capsaicin or the TRPV1 antagonist 5'-IRTX, significantly increases formalin-evoked nociceptive behaviour. These effects were not seen in WKY rats. The effects of capsaicin were likely due to TRPV1 desensitisation, given their similarity to the effects of 5'-IRTX.
- c) Demonstration that intra-VLPAG administration of capsaicin or 5'-IRTX reduces nociceptive behaviour in a moderate and transient manner in SD rats and similar effects were seen with 5'-IRTX in WKY rats. The effects of capsaicin in SD rats were likely due to TRPV1 desensitisation, given their similarity to the effects of 5'-IRTX.

- d) Demonstration that intra-LPAG administration of capsaicin or 5'-IRTX had little or no effect on formalin-evoked nociceptive behaviour in SD or WKY rats and is unlikely to make a significant contribution to the hyperalgesic phenotype of WKY rats.

In chapter 2, WKY rats exhibited anxiety-like behaviour in the open field test and EPM test, along with lower rearing and grooming activity and higher defecation when exposed to a novel environment, when compared to SD rats. WKY rats exhibited lower response latencies in the hot plate test and greater formalin-evoked nociceptive behaviour, when compared to SD rats, confirming the hyperalgesic phenotype. The qRT-PCR data indicated that TRPV1 mRNA expression was significantly lower in the DLPAG and higher in the LPAG of WKY rats compared with SD counterparts. There were no significant differences in TRPV1 mRNA expression in the VLPAG between the two strains. Intra-plantar injection of formalin significantly decreased TRPV1 mRNA levels in the DLPAG and increased TRPV1 mRNA levels in the VLPAG of SD, but not WKY rats. However, there were no significant changes in TRPV1 protein expression in any of the 3 columns of the PAG with respect to strain and/or formalin treatment. Possible reasons for the discrepancies between TRPV1 mRNA and protein levels include (1) differential temporal profiles, (2) reliability of the TRPV1 antibody, (3) translation of TRPV1 mRNA to protein might take more than 30 mins

The data presented in chapters 3-5 demonstrate that TRPV1 in the DLPAG and VLPAG regulates formalin-evoked nociceptive behaviour differentially in SD rats versus WKY counterparts and that alteration in TRPV1 expression or functionality might contribute to the hyperalgesic phenotype displayed by the stress-sensitive WKY strain. In SD rats, formalin-evoked nociceptive behaviour was associated with reduced TRPV1 expression in the DLPAG. WKY rats also had lower levels of TRPV1 expression in the DLPAG compared with SD rats and we hypothesised that this may explain their propensity to respond in a hyperalgesic manner to formalin injection. Moreover, we observed a formalin-induced increase in TRPV1 expression in the DLPAG of WKY rats and hypothesised that this may represent a compensatory change in an attempt to reduce pain behaviour in the WKY strain. To further test these hypotheses, we investigated the effects of pharmacological manipulation of TRPV1 in the DLPAG on formalin-evoked

nociceptive behaviour in both strains using the TRPV1 agonist capsaicin and the TRPV1 antagonist 5'-IRTX. In SD rats, intra-DLPAG administration of either capsaicin or 5'-IRTX had a pronociceptive effect. The effect of capsaicin was likely due to desensitisation of TRPV1 in the DLPAG, given that its effects were similar to the effects of the TRPV1 blockade with 5'-IRTX. Thus, these data are compatible with the idea that lower TRPV1 signalling in the DLPAG is associated with increased formalin-evoked nociceptive behaviour. Interestingly, the co-administration of capsaicin and 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in SD rats, likely due to both drugs competing dynamically for binding to TRPV1, with neither drug binding for long enough to desensitise (capsaicin) or block (5'-IRTX) the channel. In contrast to their effects in SD rats, intra-DLPAG administration of capsaicin or 5'-IRTX had no effect on formalin-evoked nociceptive behaviour in WKY rats. One possible explanation for these findings is that the formalin-evoked increase in TRPV1 expression in WKY rats serves to counteract/oppose the desensitisation or blockade caused by capsaicin or 5'-IRTX, respectively. Taken together, our data suggest that lower expression and/or functionality of TRPV1 in the DLPAG is associated with increased inflammatory pain behaviour and may underpin the hyperalgesic phenotype of WKY rats.

Formalin-evoked nociceptive behaviour in SD rats was also associated with higher TRPV1 expression in the VLPAG. Accordingly, capsaicin-induced desensitisation of TRPV1 in the VLPAG had an antinociceptive effect in SD rats. In contrast, WKY rats were non-responsive to intra-VLPAG capsaicin, possibly because formalin-treated WKY rats had lower TRPV1 expression in the VLPAG compared with SD counterparts. Such an effect on the TRPV1 expression in WKY rats may constitute a compensatory change to counter the hyperalgesic response to formalin exhibited by this strain. Both strains exhibited modest and transient antinociceptive effects following intra-VLPAG administration of 5'-IRTX alone, or in combination with capsaicin. In the LPAG, although saline-injected WKY rats had higher levels of TRPV1 expression compared with SD counterparts, formalin injection had no effect on TRPV1 expression and there were no effects of intra-LPAG administration of capsaicin on formalin-evoked nociceptive behaviour, in either strain. There were some modest and transient antinociceptive effects of intra-LPAG administration of 5'-IRTX alone in SD rats, but overall the data suggest that modulation of TRPV1 activity in the LPAG has little effect

on formalin-evoked nociceptive behaviour in SD rats (unlike TRPV1 in the DLPAG and VLPAG) and does not contribute to the hyperalgesic state in WKY rats. The drug treatment had no significant effect on locomotor activity/non-pain related behaviours in either strain when injected into any of the 3 columns which suggest that the effects of the drug treatments on formalin-evoked nociceptive behaviour were specific effects on nociceptive processing and were not confounded by non-specific, overt effects on locomotor activity. WKY rats exhibited less rearing behaviour when compared to SD rats as has been reported previously (Burke et al., 2010; Paré, 1994).

Sprague-Dawley rats Wistar-Kyoto rats

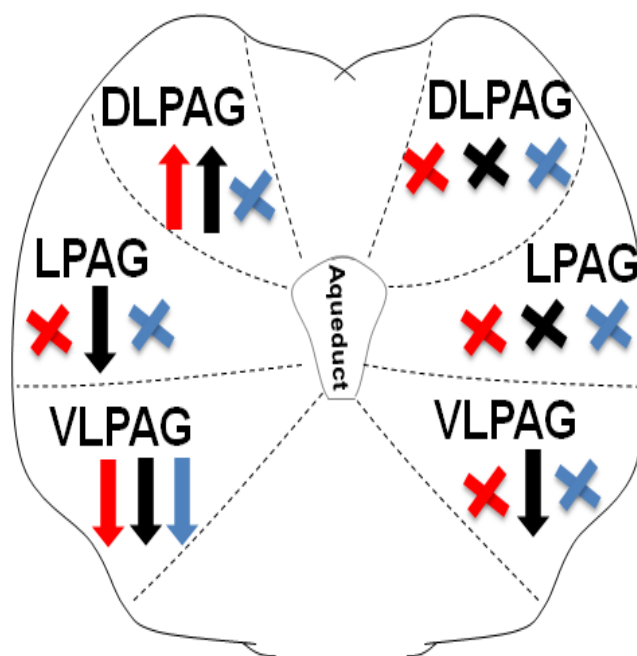


Figure 6.1: Summary of the effects of pharmacological modulation of TRPV1 in columns of the PAG on formalin-evoked nociceptive behaviour in Sprague-Dawley versus Wistar-Kyoto rats. Effects of the TRPV1 agonist capsaicin (red), the TRPV1 antagonist 5⁷-Iodoresiniferatoxin (5⁷-IRTX) (black) and combination of both capsaicin and 5⁷-IRTX (blue) on formalin-evoked nociceptive behaviour in the dorsolateral periaqueductal grey (DLPAG), lateral PAG (LPAG) and ventrolateral PAG (VLPAG) of Sprague-Dawley versus Wistar-Kyoto rats. ↑ and ↓ indicate increases and reductions in formalin-evoked nociceptive behaviour, respectively, and X indicates no effect.

The studies described herein are the first to demonstrate differential roles for TRPV1 in columns of the PAG in the regulation of formalin-evoked nociceptive behaviour in SD versus WKY rats. Previous studies focusing on thermal nociception have confirmed that TRPV1 within the PAG is involved in modulating pain behaviour. For example,

capsaicin administration into the dorsal PAG of SD rats produces pronociceptive effects in the rat tail flick test, an effect associated with increased ON cell activity in the RVM (McGaraughty et al., 2003). Our data demonstrating that capsaicin injection into the DLPAG of SD rats was pronociceptive in the formalin test supports these results and extends them in the context of inflammatory pain. Conversely, capsaicin microinjection into the DLPAG has been shown to have an antinociceptive effect in the plantar test of thermoceptive sensitivity, an effect mediated by glutamate-induced activation of mGluR₁ and NMDA receptors in the DLPAG (Palazzo et al., 2002). Administration of AA-5HT, a FAAH inhibitor and TRPV1 antagonist, into the VLPAG, was antinociceptive in the rat formalin test, an effect associated with reduced ON cell and OFF cell activity in the RVM. The PAG - locus coeruleus (LC) - spinal cord pathway was implicated in these effects (de Novellis et al., 2008). Liao et al reported that capsaicin injected into the VLPAG, increased paw withdrawal latency in the hot plate test in Wistar rats, similar to what we have reported in experiment 3 for SD rats (capsaicin microinjection into VLPAG decreased a formalin-evoked nociceptive behaviour) (Liao et al., 2011). They suggested that capsaicin activates TRPV1 on glutamatergic neurons in the VLPAG, resulting in the mGluR5-mediated production of 2-AG which in turn results in retrograde disinhibition of GABAergic neurons with consequent activation of the descending inhibitory pain pathway and antinociception (Liao et al., 2011). Intra-VLPAG microinjection of 5'-IRTX alone reduced the latency of the nociceptive reaction (pronociceptive effect) in the plantar test and this effect of 5'-IRTX was abolished when co-administered with capsaicin (Starowicz et al., 2007). The precise localisation of TRPV1 on either GABAergic or glutamatergic neurons in the PAG columns is unknown, but differential effects of modulation of TRPV1 on these neuronal subtypes is likely to dictate the effects on nociceptive behaviour.

Previous studies suggest that TRPV1 and CB₁ receptors exert opposite effects on anxiety-related behaviour (Casarotto et al., 2012a; Uliana et al., 2016) conditions. Furthermore, previous work from our laboratory suggests a differential role of the CB₁ receptor in the columns of the PAG in hyperalgesia associated with negative affective state. In SD rats, administration of the CB₁ receptor agonist arachidonyl-2'-chloroethylamide (ACEA) into either the LPAG or DLPAG (but not the VLPAG) reduced the formalin-evoked nociceptive behaviour. In WKY rats, ACEA failed to alter formalin-evoked nociceptive behaviour after administration into any of the PAG

columns, indicating a hypofunctionality of the CB₁ receptor in the LPAG and DLPAG of WKY rats (Jennings, 2015). The aforementioned data, together with the data presented in this thesis, also demonstrate that TRPV1 and CB₁ modulate formalin-evoked nociceptive behaviour in SD rats in a column-specific manner.

WKY rats are proposed as an animal model of brain–gut axis dysfunction because of dysregulation in the HPA-axis (Rittenhouse et al., 2002; Solberg et al., 2001), visceral hypersensitivity (Gibney et al. 2010; Greenwood-Van Meerveld et al. 2005) and altered colon morphology (O'Malley et al., 2010). The midbrain periaqueductal gray (PAG), which regulates anxiety and defensive behaviour as described in the introduction, has 5-HT immunoreactive cell fibers and anatomical connections with the paraventricular nucleus (PVN) of the hypothalamus (Berrino et al., 1996). In human and animal models, stimulation of this structure evokes anxiety and panic-like responses, eventually accompanied by activation of the HPA axis (Lim et al., 2011; Nashold et al., 1969). The HPA axis plays an important role in the modulation of anxiety-like behaviour (Arborelius et al., 1999; Gibbons, 1964). Earlier studies have consistently shown that a significant correlation exists between the activation of the HPA axis and stress- or anxiety-related behaviour (Smith et al., 1998). In particular, abnormal or increased function of the HPA axis has consistently been found in mood and anxiety disorders (Ströhle and Holsboer, 2003). Additionally, the HPA axis and 5-HT are reciprocally interconnected in terms of their influence on behaviour. It has been shown that activity of the HPA axis can also be stimulated by administration of 5-HT precursors, reuptake inhibitors or receptor agonists, thereby enhancing 5-HT neurotransmission and eventually increasing stress hormone levels via the PVN of the hypothalamus (Fuller and Snoddy, 1990; Heisler et al., 2007). It was shown that the PAG-PVN pathway is critical for neuroendocrine regulation of emotion (Bandler and Shipley, 1994; Lim et al., 2011). This neural circuitry of anxiety is generally affected by a group of 5-HT-containing cells in the dorsal raphe nucleus, which project to the PAG, one of the target regions for anxiolytic drugs (Graeff et al., 1993; Lowry et al., 2005). Recently, the endocannabinoid system has been implicated in genetic predisposition to depression-like behaviour in WKY rats (Vinod et al., 2012), where WKY rats had higher levels of FAAH and CB₁ in the PFC and hippocampus and lower levels of AEA and BDNF when compared to the Wistar strain. The higher levels of BDNF in PFC and hippocampus after pharmacological blockade of FAAH might contribute to the antidepressant activity

in WKY rats in the FST and sucrose preference tests (Vinod et al., 2012). Navvaria et al have shown that restraint stress in Wistar rats increases TRPV1 and FAAH mRNA and protein expression in rat hippocampal tissue. In stressed conditions, the Wistar rats reported higher corticosterone levels when compared to non-stressed controls. Administration of AA-5HT at a dose of 5mg/kg decreased corticosterone levels in the stressed rats and reversed the behavioural despair in the FST. The effects of AA-5-HT (5mg/kg) mimicked clomipramine (50mg/kg) in the FST. This study suggests that blockade of TRPV1 and FAAH reversed the behavioural despair in Wistar rats by modulating the HPA axis. In my thesis, corticosterone levels were not evaluated but based on the studies in the literature the HPA-axis might be influenced by pharmacological modulation of TRPV1 in the PAG.

As discussed earlier, the RVM is a critical component of the descending inhibitory pain pathway. Evidence for localization of TRPV1 receptors in the RVM has been provided by pharmacological studies (Starowicz et al., 2007). Application of submicromolar concentrations of anandamide and methanandamide reduced the amplitude of postsynaptic GABAergic currents in the rat brain slices, an effect which was blocked by rimonabant (Vaughan et al., 1999). Starowicz has shown that glutamate levels do effect the nociceptive behaviour at the RVM. The spinal cord is a projection target for neurons descending as part of the inhibitory pain pathway. Kim et al. 2012 proposed that TRPV1 activation induces LTD in GABAergic interneurons. The difference in the neurotransmitters and endocannabinoid levels on the ipsilateral and contralateral side might be due to formalin injection. The neurotransmitters and endocannabinoid levels were not evaluated in the dorsal horn of spinal cord and RVM after formalin injection without any i.c.v injection. Such a study might be able to evaluate the effects of formalin injection alone at the spinal cord and the RVM in both the SD and WKY rats.

Rea et al., from our laboratory, demonstrated that WKY rats have reduced formalin-evoked AEA and 2-AG levels in the RVM compared to SD counterparts. In the same study, intra-RVM administration of the FAAH inhibitor URB597 attenuated enhanced formalin-evoked nociceptive behaviour in WKY rats, suggesting impaired endocannabinoid mobilisation but not CB₁ receptor insensitivity in the RVM of hyperalgesic WKY rats (Rea et al., 2014). Jennings et al provides an evidence that the WKY model of negative affect is insensitive to a CB₁ receptor agonist administered

directly into all three columns of the PAG (Jennings, 2015). The CB₁ receptor insensitivity in the PAG of WKY rats (Jennings, 2015), might explain the downstream impaired mobilisation of endocannabinoids in the RVM (Rea et al., 2014). As the CB₁ receptors in PAG are unable to modulate RVM cell activity, then ascending pain transmission remains uninhibited through failure to engage the descending inhibitory pain pathway, explaining the enhanced formalin-evoked nociceptive behaviour of WKY rats compared to SD rats. Thus, taken together, the work presented in my thesis and the previous work on CB₁ in the PAG suggest that altered expression and/or functionality of TRPV1 and CB₁ may underly the hyperalgesic behaviour in WKY rats.

WKY-vehicle treated rats exhibited hyperalgesic behaviour compared to their SD counterparts across the intracerebral drug injection studies. But the hyperalgesic profile for WKY rats is different in each study, and this differential effect might be attributed to the cannulae received into various PAG columns or vehicle (100% DMSO) injections. The hyperalgesic behaviour had not reached the ceiling effect as in the data from our lab (Jennings, 2015) reported that WKY-vehicle treated rats exhibit higher hyperalgesic behavioural profile compared to the behavioural profiles of WKY rats in this thesis. The SD-vehicle treated rats also exhibited differential pain profiles across the intracerebral drug injection studies and this might be attributed to the aforementioned factors as well.

Pharmacological modulation of TRPV1 in the columns of the PAG had little effect on the levels of neurotransmitters and endocannabinoids/N-acylethanolamines in the RVM and dorsal horn of the spinal cord. One limitation is that these analytes were assayed at just one-time point at the end of the formalin trials when drug effects on nociceptive behaviour were often no longer apparent. Another limitation is that only tissue levels of the analytes were measured, and not the released, extracellular signalling pool which may well have been altered significantly by treatment. Studies employing *in-vivo* microdialysis should be carried out to further investigate this possibility. Intra-DLPAG or intra-VLPAG administration of capsaicin or 5'-IRTX alone or in combination with capsaicin had no effect on *c-Fos* mRNA expression in the RVM or dorsal horn of the spinal cord when compared to vehicle treatment in SD and WKY rats. In previous work from our laboratory, intra-DLPAG or intra-VLPAG administration of ACEA, while reducing formalin-evoked nociceptive behaviour (intra-DLPAG), did not alter activity-Fos expression in the dorsal horn of the spinal cord or RVM in SD rats (Jennings,

2015). These data suggest that pharmacological modulation of either TRPV1 or CB₁ receptors in the DLPAG and VLPAG can modulate formalin-evoked nociceptive behaviour without associated modulation of neuronal activity in the RVM or dorsal horn. It should, however, be noted that in the current studies only *c-Fos* mRNA was analysed and it is possible that changes may have been evident at the level of c-Fos protein.

There are some additional limitations of the studies described herein which should be noted. The intracerebral injections were administered on both the right and left sides of each PAG column, but formalin was injected only into the right hind paw. Hence the effects of pharmacological modulation of TRPV1 in the right versus left sides of each column have not been studied herein. While such a study may be of interest, we reasoned that the majority of studies of the PAG tend to target both sides simultaneously and, given the nature of the projections which converge in the RVM, it seems unlikely that TRPV1 in the ipsilateral versus contralateral sides of the PAG would play differential roles in pain-related behaviour. The very high numbers of rats that would be required also made it difficult to justify such an approach, in the context of the 3Rs. Another limitation of the studies described is that only single doses of capsaicin and 5'-IRTX were used. Again, while a dose-response component may be informative, it was difficult to justify given that (a) similar studies have already been published in other models of pain and provided a strong basis for choosing the doses used in the present studies and (b) the 3Rs and the large number of rats that would have been required to run dose-response studies in both rat strains. Other limitations relating to the discrepancies between mRNA versus protein expression for TRPV1 expression, and the lack of effects on neurotransmitter and *c-Fos* levels have already been discussed above.

The work described in this thesis provides a firm foundation for future studies aimed at further elucidating the role of supraspinal TRPV1 in hyperalgesia associated with negative affective state. This work could be broadly classified as preclinical and clinical and some suggestions for future work include the following:

- a) Determine whether TRPV1 is localised on GABAergic or glutamatergic neurons in the specific columns of the PAG to further aid our understanding of the neuronal mechanisms by which TRPV1 in the PAG modulates pain- and anxiety-related behaviour.
- b) Electrophysiological studies at the level of PAG, RVM and spinal cord to further examine whether pharmacological modulation of TRPV1 in the PAG columns engages descending pain pathways in formalin-injected SD vs WKY rats.
- c) Microdialysis studies monitoring the levels of GABA and glutamate in key regions such as RVM or spinal cord after administration of TRPV1 agonist and antagonist into the specific columns of the PAG to enhance our understanding of TRPV1-mediated neurochemical effects.
- d) Investigation of the potential role of TRPV1 in other brain regions (e.g. amygdala, medial prefrontal cortex).

Clinically, the role of TRPV1 in hyperalgesia associated with negative affect hasn't been evaluated. Demonstration of such a role in humans would support translatability of the results that have been shown in pre-clinical studies. Using techniques such as qRT-PCR and western blotting, it would be possible to determine the expression of TRPV1 in key neural substrates such as PAG, mPFC, amygdala involved in modulating pain states in post-mortem tissue from the patients with co-morbid anxiety and pain disorders. In addition, plasma and cerebrospinal fluid from such patients could be assayed for endocannabinoids and neurotransmitter levels and their responses to TRPV1 agonist and antagonist administration could be investigated.

In conclusion, the results of this thesis have shown that modulation of inflammatory pain by TRPV1 in the PAG occurs in a column-specific manner. The data also provide evidence for differences in the expression of TRPV1, and differences in the effects of pharmacological modulation of TRPV1 in specific PAG columns, between WKY and SD rats, suggesting that TRPV1 expression and/or functionality in the PAG plays a role in hyper-responsivity to inflammatory pain in a genetic background prone to negative

affect. These findings may have implications for the understanding and treatment of persistent pain that is co-morbid with or exacerbated by, affective disorders.

Appendix

A. Buffers and Solutions

1.1 Western Immunoblotting

4X separation buffer (for 100ml)

- 1.5M Tris: 18.2g
- 4ml of 10% Sodium dodecyl sulphate (SDS; Sigma-Aldrich, Ireland, 20% solution diluted to 10%)
- Fill to final volume minus SDS as SDS forms bubbles, then add SDS)
- pH 8.8 with HCl

4X Stacking gel buffer (for 100ml)

- 0.05 M Tris: 6g
- 0.4% SDS: 4ml of 10% SDS (Sigma-Aldrich, Ireland, 20% solution diluted to 10%):
- pH 6.8 with HCl

10X Running buffer (for 1L)

- Glycine (Sigma-Aldrich, Ireland): 144g
- Trizma-Base (Sigma-Aldrich, Ireland): 30g
- 100ml of 10 % SDS
- Dissolve in 1L of distilled water

10 X Transfer buffer

- Glycine (Sigma-Aldrich, Ireland): 144g
- Trizma-Base (Sigma-Aldrich, Ireland): 30g
- Make up to 1L distilled water

Making 8% separation gel for 2 gels

- 30% acrylamide (Sigma, Ireland): 5.3ml
- 4x sep buffer: 5ml
- Distilled water : 9.5ml
- 10% ammonium persulphate (Sigma, Ireland): 200 μ l

- TEMED (Sigma-Aldrich, Ireland) (in fume hood) : 20 μ l

Stacking gel for 10 ml (2 gels)

- 30% acrylamide (Sigma, Ireland): 1ml
- 4x stacking buffer: 2.5ml
- Distilled water : 6.5ml
- 10% ammonium persulphate (Sigma-Aldrich, Ireland): 100 μ l
- TEMED (Sigma-Aldrich, Ireland) (in fume hood) : 10 μ l

10x Tris-buffered saline (TBS) (1L)

- 200mM Trizma-Base (Sigma-Aldrich, Ireland), Ireland): 24.23g
- 1.37M NaCl (Fisher, Ireland): 80.06g
- Distilled water: 800mL
- Ph to 7.6 with HCL
- Make up to 1L with distilled water

Blocking solution: 5% non-fat dry milk (Aptamil, UK) in 0.05% Tween-20 (Sigma, Ireland) TBS

Primary Antibody: polyclonal mouse antibody to the TRPV1 (N-term) (1:200, catalogue no.75254;Antibodies Inc, USA) and mouse monoclonal antibody to b-Actin (1:10,000, A5441; Sigma-Aldrich, Ireland) diluted in 5% milk/0.1% Tween PBS.

Secondary antibody: IRDye conjugated goat anti-mouse (k700; (1:10,000; catalogue no. 926-68020; LICOR Biosciences, UK) in 1% milk/0.05% Tween-20 TBS.

B. Supplementary data

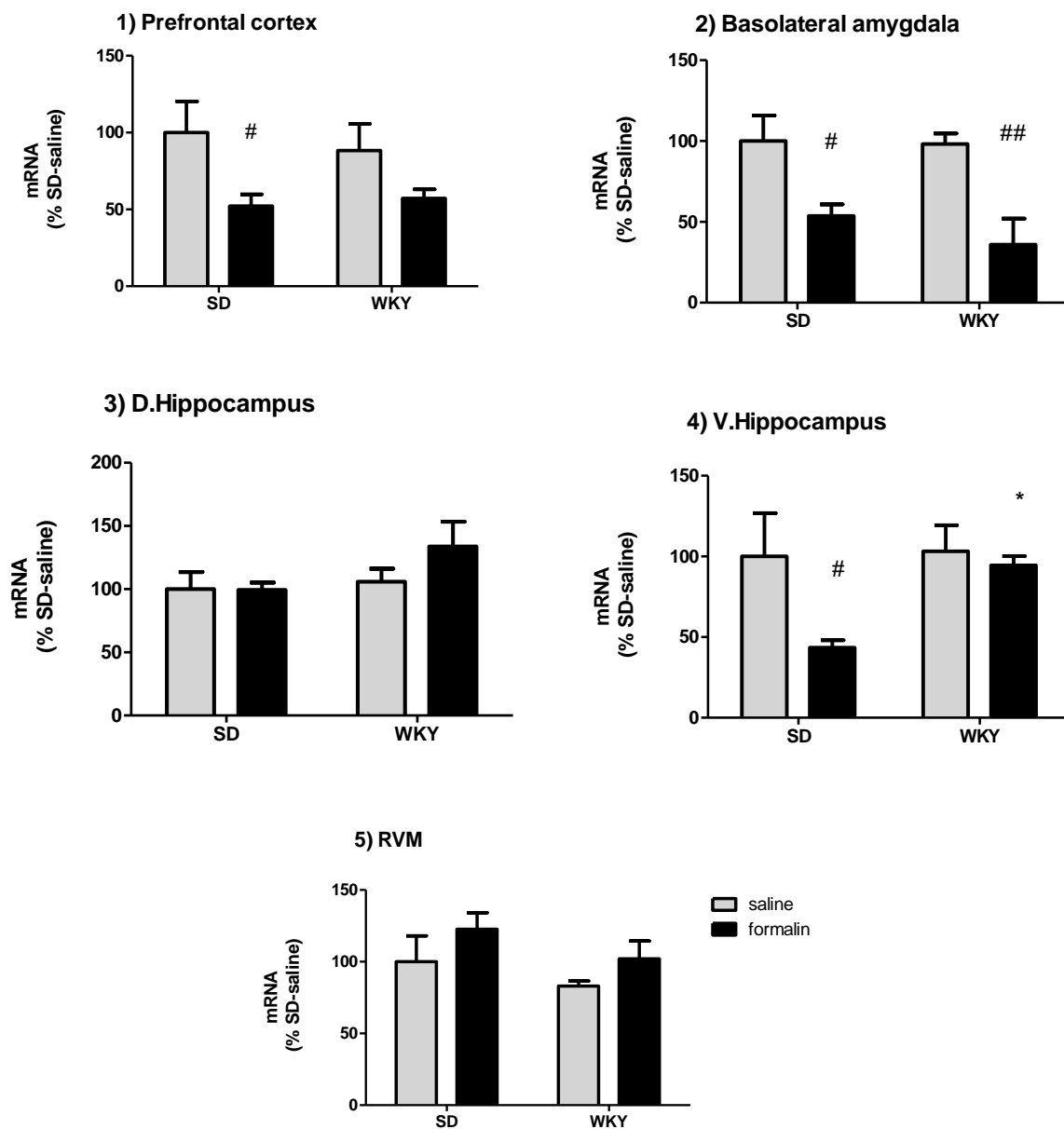


Fig 1:TRPV1 mRNA levels in the key brain regions and spinal cord of SD and WKY rats that received intra-plantar injection of either saline or formalin. (1) Prefrontal cortex: Two-way ANOVA effects of strain: $F_{1,22} = 0.059$, $P = 0.811$; formalin: $F_{1,22} = 8.381$, $P < 0.01$ and strain \times formalin interaction: $F_{1,22} = 0.380$, $P = 0.545$ followed by Fisher's LSD post hoc test ($\#P < 0.05$, vs SD-SAL). (2) Basolateral amygdala: Two-way ANOVA effects of strain: $F_{1,19} = 0.623$, $P = 0.441$; formalin: $F_{1,19} = 19.077$, $P < 0.001$ and strain \times formalin interaction: $F_{1,19} = 0.411$, $P = 0.531$ followed by Fisher's LSD post hoc test ($\#P < 0.05$, $\#\#P < 0.01$ vs respective SAL treatment). (3) Dorsal Hippocampus: Two-way ANOVA effects of strain: $F_{1,21} = 2.700$, $P = 0.118$; formalin: $F_{1,21} = 1.244$, $P = 0.279$ and strain \times formalin interaction: $F_{1,21} = 1.341$, $P =$

0.262). (4) Ventral Hippocampus: Two-way ANOVA effects of strain: $F_{1,22} = 3.338$, $P = 0.083$; formalin: $F_{1,22} = 4.860$, $P < 0.05$ and strain \times formalin interaction: $F_{1,22} = 2.602$, $P = 0.123$) followed by Fisher's LSD post hoc test ($^{\#}P < 0.05$, vs SD-SAL treatment; $^*P < 0.05$, vs SD-Form treatment). (e) Dorsal Horn of spinal cord: Two-way ANOVA effects of strain: $F_{1,22} = 2.651$, $P = 0.121$; formalin: $F_{1,22} = 3.261$, $P = 0.088$ and strain \times formalin interaction: $F_{1,22} = 0.026$, $P = 0.873$).

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