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Is postoperative hypernociception associated with anxiety-like behaviour in rats?

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Is postoperative hypernociception associated with anxiety-like behaviour in rats?

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, South Africa, 2013

DECLARATION

I, Stephanie Ferreira declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

..... [Signature of candidate]

.....day of.....[month], 20....

I certify that the studies contained in this dissertation have the approval of the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg. The ethics approval numbers are 2010/20/04 and 2011/12/04.

..... [Signature of candidate]

.....day of.....[month], 20...

CONFERENCE PROCEEDINGS AND PRESENTATIONS

Data presented in this dissertation have been presented in the form of oral presentations at:

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ABSTRACT

Existing animal models of postoperative pain have focused on the sensory aspects of postoperative nociception and have ignored the affective components of pain, such as anxiety, which in human studies have been shown to be important determinants of the overall pain experience and pain outcomes. Therefore, I investigated whether anxiety-like behaviour in rats was a feature of an established animal model of postoperative pain. Postoperative hypernociception was assessed on a daily basis prior to surgery and nine days after surgery in 10 male Sprague-Dawley rats, that had had an incision made through the abdominal wall. Nociceptive thresholds were tested using an anaesthesiometer, which was applied to the wound until the rat showed aversive responses. Anxiety-like behaviour was assessed in a separate group of 50 experimental and 50 control rats that had undergone the same surgical intervention or sham surgery (anaesthesia only). The open field paradigm was used to test anxiety-like behaviour and involved placing rats in a 1 m² arena and measuring their exploratory behaviour; behaviour that is reduced in anxious rats. Additional 40 experimental and 40 control rats were decapitated and trunk blood was collected for corticosterone measurement, and the prefrontal cortices and hippocampi were excised for measurement of monoamines, including serotonin, noradrenaline and dopamine, as well as the neurotransmitters GABA and glutamate on postoperative days one, two, four and nine. Surgery produced a significant decrease in nociceptive thresholds for up to six days, however there was no significant decrease in exploratory behaviours between control and surgery rats at any stage after surgery. There was also no significant difference between the monoamines, GABA, glutamate or corticosterone levels between the surgery and control groups, on any of the postoperative days I assessed. However, a

significant increase in dopamine concentrations in sham surgery rats compared to control and surgery groups was found. It therefore appears that, in an established model of postoperative pain, rats do not display anxiety-like behaviour, or express circulating or brain biomarkers of stress.

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LIST OF ABBREVIATIONS

5HT	Serotonin
AMPA/KA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid/Kainate
BDNF	Brain derived neurotrophic factor
BZD	Benzodiazepine
CINC-1	Cytokine-induced neutrophil chemoattractant-1
CORT	Corticosterone
COX	Cyclooxygenase enzyme
DA	Dopamine
DHN	Dorsal horn neuron
EAA	Excitatory amino acid
GABA	Aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HT	High threshold
HTTLPR	5-HydroxyTryptamine Transporter Gene-Linked Polymorphic Region
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
LC	Locus coeruleus
MAO-A	Monoamine oxidase-A
mGluR	Metabotropic glutamate receptors
mPFC	Medial prefrontal cortex
NA	Noradrenaline

NAc	Nucleus accumbens
NMDA	N-Methyl-D-aspartate
NSB	Non-specific binding
PGE ₂	Prostaglandin E ₂
PFC	Prefrontal cortex
RAIC	Rostral agranular insular cortex
RF	Receptive field
RIA	Radioimmunoassay
SERT	Serotonin transporter
SytIV	Synaptotagmin IV
TRPV ₁	Transient receptor potential vanilloid 1
TNF- α	Tumor necrosis factor- α
WDR	Wide dynamic range
WT	Wild type

Chapter 1

Introduction

1.1 Postoperative Pain

At least two-thirds of patients that undergo surgery experience moderate to severe pain postoperatively despite analgesic therapy (Apfelbaum, et al., 2003). Postoperative pain may be spontaneous or evoked by movements such as coughing and applying pressure to or near to the injured area (Woo, et al., 2004). Poor management of postoperative pain is associated with increased mortality, along with complications such as poor wound healing, insomnia, deep vein thrombosis and myocardial infarction (Carr & Goudas, 1999). Poorly controlled postoperative pain is also associated with the extension of hospital stay and an increase in hospital readmissions, along with increased risk of developing chronic pain, which in turn increases health care costs (Twersky, et al., 1997; Cousins, et al., 2000; Martin, et al., 2005). Poor postoperative pain control may reflect poor implementation of postoperative pain management guidelines by health care professionals, but also could be due to inadequate understanding of the pathophysiology of postoperative pain, and hence poor ability to implement effective treatments (Apfelbaum, et al., 2003). It is therefore important to do research on postoperative pain using animal and human models (Banik & Brennan, 2004; Pogatzki, et al., 2005) and study the efficacy of analgesics to treat this form of pain (Brennan, et al., 1996).

Efforts to improve knowledge of the pathophysiology of postoperative pain and to test novel therapeutic agents include the use of animal models of surgical pain (Whiteside, et al., 2004). Typically, animal models of postoperative pain involve surgical incisions through the skin and underlying fascia of the rat paw (i.e. plantar incision) (Brennan, et al., 1996), gastrocnemius muscle (Pogatzki, et al., 2003), tail (Weber, et al., 2005; Loram, et al., 2007) or abdomen (i.e. laparotomy) (Martin, et al., 2004; Brennan, et al., 2005), and

measuring the animal's response to a punctuate or blunt noxious mechanical stimulus applied to the wound. In general, the hypernociception induced in these models mimics the duration of mechanical hyperalgesia seen in the human postoperative period (Brennan et al., 1996; Pogatzki, et al., 2002b). In this introductory chapter I will firstly discuss the mechanisms identified, using these animal models as underlying postoperative pain, including the role of primary and secondary sensitization and the involvement of prostaglandins, interleukins and cyclooxygenase enzyme (COX). Thereafter, I will focus on the affective component of pain including discussions on studies that investigated the relationship between pain and anxiety, and also animal models that typically are used in the investigation of anxiety-like behaviour in rats. Lastly, I will focus on the brain and the neurotransmitters that are believed to be involved in the modulation of anxiety and pain, namely, serotonin (5HT), noradrenaline (NA), dopamine (DA), γ -aminobutyric acid (GABA), as well as the stress hormone, corticosterone (CORT).

It is important to note some of the nomenclature used in this dissertation. It is easy to confuse the term pain and nociception and one should note they are not the same (Loeser & Treede, 2008). Pain is a subjective phenomenon whereas nociception refers to the neuronal processing which occurs in response to a noxious stimulus (Loeser & Treede, 2008). When referring to increased pain sensation in humans when a noxious stimulus is applied to a wound, the term 'hyperalgesia' will be used in this dissertation. However, it is not known whether rats experience the subjective feeling of "pain" as humans do, and so their increased "pain" sensation is referred to as hypernociception and relates to enhanced behavioural or physiological response to a noxious stimulus (Loeser & Treede, 2008).

1.1.1 Mechanisms of postoperative pain

Primary hypernociception, secondary hypernociception and allodynia have been observed in studies of postoperative pain (Kawamata, et al., 2005). Primary hypernociception refers to an increased nociceptive response to noxious stimuli applied to the injured area while secondary hypernociception refers to increased nociceptive responses to stimuli applied to the undamaged area surrounding the injury site (Zahn et al., 1999b; 2002b; Pogatzki, et al., 2003). Allodynia refers to an increased nociceptive response to non-noxious stimuli (LoPinto, 2006; Loeser & Treede, 2008). Peripheral and central sensitization of nociceptive pathways, mediate these heightened nociceptive states after an incision (Zahn, et al., 1999b; 2002a; Pogatzki, et al., 2002c; Shavit, et al., 2005). In general, peripheral sensitization is characterized by a decreased response threshold to stimuli and an increase in spontaneous activity in these neurons (Yue, 2007). Central sensitization, on the other hand, is characterized by increased excitation of neurons within the central nervous system (Yue, 2007). It has been proposed that primary hypernociception is caused by the sensitization of primary afferent fibers (peripheral sensitization) while secondary hypernociception is caused by sensitization of spinal dorsal horn neurons (central sensitization) (Zahn, et al., 1999a; 2002a; Pogatzki, et al., 2002c; Shavit, et al., 2005). Peripheral and central sensitization has been studied in incision animal models of postoperative pain along with inflammatory and neuropathic models. However, there are features of these sensitization processes that are unique to models of postoperative pain, which will be discussed in more detail below.

1.1.1.1 Central sensitization

Central sensitization involves altered activity within the spinal cord (Vandermeulen & Brennan, 2000). Many rodent spinal hypernociceptive studies have been conducted on wide dynamic range (WDR) neurons and high threshold (HT) neurons, which are both dorsal horn neurons (DHN) and differ according to their responses to the stimuli evoked (Tsuruoka, et al., 2008). For example, HT neurons only respond to input from primary afferents stimulated by noxious mechanical stimuli such as strong pinch stimuli, whereas WDR neurons respond to input from primary afferents stimulated by innocuous and noxious mechanical stimuli, such as brush and pinch stimuli (Tsuruoka, et al., 2008). A study by Vandermeulen and Brennan (2000), revealed that WDR neurons demonstrated an increase in background activity along with expanded receptive fields (RF) after a plantar incision was made to the rat hindpaw. The result was not found in HT neurons. The same rats that underwent the plantar incision displayed increased withdrawal responses to noxious stimuli, indicating mechanical hypernociception. It was therefore concluded that altered activity in WDR neurons and not HT neurons is involved in postoperative mechanical hypernociception in rats (Vandermeulen & Brennan, 2000). Another study supporting this finding, demonstrated that WDR neurons become hyperexcitable in response to noxious and innocuous stimuli when an incision was made in the hairy skin of the rat, but HT neurons did not (Kawamata, et al., 2005). Moreover, the RF size remained the same in HT neurons after a response to high-threshold stimuli. However, when the incision was made in the glabrous skin, the RF size increased in some HT neurons (Kawamata, et al., 2005a), indicating a possible difference in mechanism between glabrous and non-glabrous skin. Whereas, other studies have suggested that mechanically insensitive areas of the RF of WDR neurons that become mechanically sensitive after

plantar incision are involved in mechanical nociception which is demonstrated in the rat model (Zahn, & Brennan, 1999a).

Altered activity in spinal neurons is influenced by the release of excitatory amino acids (EAA). It has been shown that after a plantar incision in male Sprague-Dawley rats there is an increase in glutamate and aspartate (EAAs) in the dorsal horn, which returns to pre-incision levels one hour later (Zahn, et al., 2002a). It is believed that this increase is caused by increased activity of primary afferent fibres following a skin incision, which then release more neurotransmitter in the dorsal horn (Zahn, et al., 2002a). Therefore increased EAA in dorsal horn neurons may be involved in the initial stages of hypernociception caused by an incision (Zahn, et al., 2002a). Accordingly, NMDA and non-NMDA receptors which are targets for glutamate, have also been studied in central sensitization. Antagonists of the NMDA receptor have been found to have an antinociceptive effect on postoperative, inflammatory and neuropathic pain states in both animal and human models (Price, et al., 1994; Sindrup, et al., 1999; Dickenson et al., 2004; D'Mello & Dickenson, 2008). In a study by Pogatzki, *et al* (2000), intrathecal administration of an NMDA receptor antagonist before a plantar incision was made, did not prevent increased pain responses to mechanical stimuli postoperatively (Pogatzki, et al., 2000). However, when a non-NMDA receptor antagonist was administered, it did reduce the pain responses to the mechanical stimuli (Pogatzki, et al., 2002b), suggesting that non-NMDA receptors are involved in the central sensitization process in postoperative hypernociception. The authors however suggested that a plantar incision was perhaps not sufficient enough to activate the spinal NMDA receptors. In a similar study, a non-NMDA receptor antagonist reversed the secondary mechanical hypernociception after gastrocnemius incision, by decreasing the dorsal horn neuron RF size, while the NMDA

receptor antagonist decreased the nociceptive behaviours (Pogatzki, et al., 2003), suggesting that different incision locations may indeed have different mechanisms contributing to central sensitization.

1.1.1.2 Peripheral sensitization

Studies of peripheral sensitization following a skin incision in rats have shown that peripheral sensitization is characterized by a decreased response threshold to stimuli in primary afferent neurons and increase in spontaneous activity in these neurons (Yue, 2007). Pogatzki, *et al* (2002), found that mechanical stimuli applied to the wound caused by plantar incision in male Sprague-Dawley rats, caused the activation and sensitization of both A δ and C-fibers, one day after a plantar incision. In addition, a study by Banik and Brennan (2004) revealed that C-fibres near the incision site showed spontaneous activity following a glabrous skin incision and were sensitive when heat stimuli were applied, but not when mechanical stimuli were applied (Banik & Brennan 2004).

Other studies have shown that factors such as changes in pH and peripheral algogenic mediators such as nerve growth factor (NGF), substance P, calcitonin gene-related peptide, 5HT, bradykinin and potassium adenosine triphosphate may also be involved in the development and maintenance of hyperalgesia following a surgical incision (Woo, et al., 2004; Banik, et al., 2005). Woo *et al* (2004) demonstrated that the pH in the gastrocnemius muscle (incision site) remained decreased after incision for the same amount of time as the nociceptive behaviours that were evident in the same rats, suggesting that a decrease in pH may be involved in hypernociception following plantar incision (Woo, et al., 2004).

Moreover, human studies involving the administration of low-pH solutions demonstrate pain along with mechanical and thermal hypernociception (Steen, 1993). A study by Banik *et al* (2005), demonstrated that there is an increase in NGF in the glabrous skin after an incision in the plantar aspect of the rat hindpaw and when anti-NGF was administered to the rodents, it decreased the hypernociceptive response to heat stimuli substantially, one and two days after incision. In another study of heat and mechanical hypernociception in a mouse model of plantar incision, using knock-out mice, it was found that the transient receptor potential vanilloid 1 (TRPV₁) was involved in heat hypernociception, but not mechanical hypernociception (Pogatzki- Zahn, et al., 2005). The paw withdrawal latencies of TRPV₁ KO mice to heat stimuli (a radiant lamp was focused from beneath a pre-warmed glass platform) were significantly longer than those of WT (wild type) mice, suggesting that heat hyperalgesia after plantar incision is prevented in TRPV₁ KO mice. However, for mechanical stimuli (calibrated von Frey filaments applied adjacent to plantar incision), paw withdrawal frequencies between TRPV₁ KO mice and WT mice were similar and both were increased one day after surgery, suggesting that TRPV₁ is involved in mechanical hyperalgesia in rats (Pogatzki-Zahn, 2005). Although my study only focuses on mechanical hyperalgesia, it is important to note that both mechanical and thermal hyperalgesia develops in postoperative pain and they have different mechanisms as can be seen by the above mentioned studies.

1.1.2 Mediators of postoperative pain

1.1.2.1 Cyclooxygenase enzymes and prostaglandins

After inflammation and damage to peripheral tissues, there is an increase in spinal cord cytokines, COX enzymes and prostaglandins (Kroin, et al., 2004). These mediators have been extensively studied in models of postoperative pain. In a study by Zhu *et al* (2003), COX-1 was increased in the spinal cord and gracile nucleus after paw incision. The increase in COX-1 within the spinal cord may be caused by substance P or glutamate, which acts on AMPA/KA receptors in the spinal cord (Zhu, et al., 2003). Furthermore, intrathecal administration of two different COX-1 inhibitors and a COX-2 inhibitor, resulted in a reduction, but not a reversal, of hypersensitivity by both COX-1 inhibitors, but not the COX-2 inhibitor. It is therefore plausible to suggest that the COX-2 enzyme may not be involved in mechanical hypersensitivity (Kamerman, et al., 2007). On the other hand, a study by Kroin *et al* (2004) demonstrated that after paw incision, an increase in COX-2 protein was measured within the lumbar region of the rat spinal cord but not the cervical region (Kroin, et al., 2004). However, this post-incisional increase in COX-2 protein was short lived as it only lasted for a few hours.

Prostaglandin receptors have been divided into subtypes namely EP₁, EP₂, EP₃ and EP₄. In a study by Omote *et al* (2001), an EP₁ receptor antagonist, administered into the plantar surface of the hindpaw on the ipsilateral side to the incision, was used to investigate the effect of this prostaglandin on peripheral sensitization in a rat plantar hindpaw incisional model (Omote, et al., 2001). The authors found that the antagonist reduced postoperative

mechanical hypernociception in a dose-dependent and time-dependent manner, when the hypernociception was tested using punctate (sharp instrument) and non- punctate stimuli. These findings suggest that the EP₁ receptor may be involved in postoperative mechanical hypernociception (Omote, et al., 2001). Of the prostaglandins, the most commonly studied is prostaglandin E₂ (PGE₂). It has been indicated that during surgical stress an increase in PGE₂ occurs within the amygdala, a brain region believed to play a role in pain caused by inflammation (Shavit, 2005). Shavit *et al* (2005) found that after an abdominal incision in male Fischer rats, there was an increase in amygdala PGE₂ concentration, therefore suggesting that PGE₂ found within the amygdala may be involved in incision-induced postoperative hypernociception (Shavit, et al., 2005).

1.1.2.2 Cytokines

Proinflammatory cytokines, such as, interleukin-1 β (IL-1 β), interleukin-6 (IL-6), cytokine-induced neutrophil chemoattractant-1 (CINC-1) and tumor necrosis factor- α (TNF- α) are involved in pain sensitization (Kawasaki, et al., 2008). Cytokines act peripherally by sensitizing nociceptors and have been found to be induced in glial cells during pain conditions, therefore acting centrally as well (Kawasaki, et al., 2008). In a study by Loram *et al* (2007), which employed an incision in the rat tail, the concentration of IL-1 β , IL-6, TNF- α and CINC-1 increased at the injury site but not in the circulation (Loram, et al., 2007). The cytokine concentration increase also occurred after the onset of hypernociception in these rats (Loram, et al., 2007) and therefore the authors suggested that the cytokines are not involved in the early stages of primary hypernociception (Loram, et al., 2007). Moreover, the authors suggested that some of these cytokines may be involved in maintaining the hypernociception for a period, as IL-1 β and IL-6

concentrations remained elevated in the circulation for the duration of the hypernociception (Loram, et al., 2007). Another study has shown that when IL-1 β is administered intrathecally in rats it produces hypernociception (Choi, et al., 2003). In addition, when administering an IL-1 β antibody in a rat, the inflammatory hypernociception caused by zymosan hind paw injection was reduced (Milligan, et al., 2003). In a study by Fu et al (2006), a deep thoracic incision in male Wistar rats caused the activation of microglia in the dorsal and ventral horns of the spinal cord, which was associated with an increase in IL-1 β in the anterior horn and ventral horn of the spinal cord (Fu, et al., 2006). The authors suggested that both the activated glial cells and increased IL-1 β in the spinal cord may contribute to hypersensitivity induced by thoracic incisions (Fu, et al., 2006).

Thus far I have reviewed the basic mechanisms which have been shown primarily in animal studies, to underlie postoperative hypernociception. However, animal models are limited because they only study spinal reflex responses to noxious stimuli applied to a wound, and do not assess higher-order nociceptive processing (Wallace, et al., 2005; 2008). Novel nociceptive-testing paradigms have been developed that assess more complex pain-like behaviours such as anxiety-like behaviour. However, investigations of complex psychomotor changes associated with hypernociception have been largely limited to studies on animal models of neuropathic pain. Neuropathic pain (i.e. damage to sensory nerve fibres) is believed to have different physiological mechanisms to those of postoperative pain (Zahn, et al., 2002). Therefore it is important to study anxiety-like behaviour in the postoperative pain animal model too. And so, my dissertation will now focus on anxiety and its involvement with postoperative pain.

1.2 Anxiety and postoperative pain

Pain after surgery is often accompanied by or affected by psychological disorders such as depression and anxiety (Ward-McQuaid, et al., 1973; Ploghaus, et al., 2001; Khan, et al., 2011). Anxiety is defined as a feeling of uneasiness towards a possible dangerous situation (Ploghaus, et al., 2001). However, this feeling of uneasiness is believed to be an adaptive response to the perceived danger and therefore provides an animal's first line of defence. Anxiety that becomes excessive may be maladaptive and often is referred to as pathological anxiety (Belzung, 2001; Cryan & Holmes, 2005; Kim & Gorman 2005). Anxiety is divided into two categories: state and trait anxiety (Belzung, 2001; Gross & Hen, 2004). In order to fully understand studies of anxiety, it is important to distinguish between the two categories. State anxiety is immediate anxiety experienced at a particular moment while trait anxiety tends to be permanent from moment to moment and is more a general feeling of fearfulness (Belzung & Griebel, 2001; Caumo, et al., 2001; Gross & Hen, 2004).

The relationship between postoperative pain and anxiety has yet to be fully understood (Caumo, et al., 2001). It has been suggested that anxiety may increase pain in the postoperative setting, however this may depend on the type of anxiety present, the type of pain present, the surgical procedure performed and the method employed to measure the pain and anxiety (Perry, et al., 1994). In human studies, preoperative anxiety has been shown to increase postoperative pain, prolong hospital stays and increase the need for analgesics following surgery (Perry, et al., 1994; Caumo, et al., 2001). In a study conducted by D'Angelo *et al* (2010), it was found that subjects who suffered from anxiety

before lumbar disc surgery were at an increased risk to suffer from pain after surgery. Another study showed that when an analgesic, (pentazocine) and an anxiolytic (oxypertine) were used in combination to treat mild and severe postoperative pain, they were more efficient in alleviating the pain than when pentazocine was used alone (Ward-McQuaid, et al., 1973). The relationship between postoperative pain and anxiety is therefore important as is the treatment of these co-morbidities. Further brain studies have shown that the hippocampus may play a role in pain-related anxiety (Ploghaus, et al., 2001). Because the hippocampus seems to be involved in pain processing, Ploghaus *et al* (2001) hypothesized that anxiety-induced hyperalgesia may be linked to the hippocampus, which is the area stimulated through experimental nociception (Ploghaus, et al., 2001). However the hippocampus is not the only brain region to be associated with pain and anxiety. Another site linking pain and anxiety is the amygdala (Guangchen, et al., 2007). It is believed that the central nucleus of the amygdala integrates information from the fear-anxiety centre with nociceptive information from the spino-parabrachio-amygdaloid pain pathway (Guangchen, et al., 2007).

It can therefore be concluded that animal and clinical studies have shown an association between pain and anxiety. To further add to the anxiety research, I will now discuss the animal models used to test anxiety, along with the anxiety biomarkers typically measured in rat brain and blood tissue.

1.3 Animal models of anxiety

In order to increase our understanding of the mechanisms underlying the relationship between postoperative pain and anxiety and to develop effective treatments to reduce the symptoms, it is first necessary to develop appropriate models that incorporate assessment of anxiety (Cryan & Holmes, 2005). Countless animal behavioural models have been developed for the assessment of anxiety in rodents, with the most common being the elevated plus maze, light/dark box and the open field tests (Clement & Chapouthier, 1997; Prut & Belzung, 2003). These exploration tests create a conflict situation between the animal's natural drive to explore a novel environment and its innate drive to avoid potentially dangerous, openly lit spaces (Mallo, et al., 2007; Bosch, et al., 2009). These open spaces are found in the form of the light compartment in the light/dark box test, the centre of the arena in the open field paradigm and the open elevated arms in the elevated plus maze, (Cryan, & Holmes, 2005).

The elevated plus maze (Cryan & Holmes, 2005) consists of two open arms and two closed arms joined at right angles to each other in a form of a cross. These arms are elevated off the floor. Generally, anxiety-like behaviour is displayed when rats tend to avoid the dangerous open arms of the elevated plus maze and spend less time exploring these arms than the closed arms (Harvey, et al., 2006). The light/dark box test (Barbier & Wang, 2009), on the other hand, consists of a light and dark compartment of the same dimensions that are separated with a transparent wall with an open door. The dark compartment has a roof, while the light compartment is transparent to light. Here, anxiety-like behaviour is displayed when rats tend to spend less time exploring the light compartment or avoid the

light compartment (Barbier & Wang, 2009). The open field paradigm (see fig. 2 in chapter 2) consists of an arena with walls (to prevent the animal from escaping) in which a rat is placed for a certain amount of time and allowed to explore the novel environment (Belzung, 2001). Rats displaying an anxiety-like behaviour tend to avoid the lit centre of the arena, therefore displaying thigmotactic behaviour, in which they stay in close proximity to the walls of the open field, where it is thought that they feel protected (Barbier & Wang, 2009). Thus, anxiety-like, thigmotactic behaviour is indicated by a decrease in the amount of entries into the centre of the arena, a reduction in the time and distance spent in the centre of the arena and an increase in the latency to enter the centre of the arena (Prut & Belzung, 2003). Anxious rodents also tend to increase their defecation, reduce their motor activity and grooming as well as rearing behaviour (Katz, et al., 1981). If food is introduced into the arena, anxious rats tend to eat less, showing a decrease in food directed behaviour (Slawecki, et al., 2003). Through the years many forms of the open field have emerged, differing in the shape (circular, square, rectangular), the position of the lighting (from above, or just illuminating the room), objects placed in the arena (platforms, columns, tunnels, food) and the experimental animal used in the test (pigs, rabbits, lambs, rats) (Prut & Belzung, 2003; Slawecki, et al., 2003).

Although animal models of anxiety are extensively used to test the efficacy of anxiolytic drugs (Prut & Belzung, 2003), in order for the model to be successful in relation to human behaviour, it has to withstand three main criteria: predictive, construct and face validity (Prut & Belzung, 2003). Predictive validity can be claimed when anxiolytics used in the clinical setting are effective in decreasing the anxiety-like behaviour within the behavioural paradigm (Belzung, 2001; Cryan & Holmes, 2005). Face validity refers to the anxiety response that an animal shows, having similarities to the anxiety response found in

the human. In the open field we observe this as the avoidance of ‘dangerous’ places, as is seen with humans (Belzung, 2001). Construct validity refers to when the model correlates with the biological mechanisms thought to underlie the clinical state (Belzung, 2001). However, behavioural models are not the only studies that are undertaken when investigating anxiety-like behaviour. Since the 1980’s, studies have focussed on the involvement of brain neurotransmitters in the modulation and regulation of anxiety and its related disorders.

1.4 Biomarkers of anxiety

1.4.1 Serotonin

5HT is a neurotransmitter acting through receptors found throughout the brain (Gordon & Hen, 2004). It has a role in early brain development (Patterson, et al., 2005) and in the control of nociception, aggression and responses to stress (Parks, et al., 1998).

Psychological stress, for example produced by electric foot shock as part of conditioned fear stress, is believed to increase 5HT release within the prefrontal cortex (PFC) and amygdala (Yoshioka, et al., 1995), while placing a rat in the elevated plus maze has also been shown to increase 5HT in the hippocampus (Kagamiishi, et al., 2003).

One view is that an increase in 5HT is related to increased anxiety (Clement & Chapouthier, 1997; Yoshioka, et al., 1995; Kagamiishi, et al., 2003). It has also been shown that drugs which block 5HT activity are anxiolytic (Holmes, et al., 2003b). In support of

this view, studies have shown that when benzodiazepines are used as anxiolytics, they reduce 5HT neurotransmission and in turn decrease anxiety (Gordon & Hen, 2004). Also, in behavioural tests such as the punished responding test, which causes an increase in 5HT levels and anxiety, using GABA microinjections into the dorsal raphe nucleus causes an anxiolytic effect, as GABA inhibits 5HT neurons (Gordon & Hen, 2004). Other studies have shown that an acute administration of 5HT is anxiogenic (Gordon & Hen, 2004). Monoamine oxidase-A (MAO-A) is an enzyme that degrades 5HT and when MAO-A deficient mice are used in studies, an anxiogenic profile is observed with an increase in 5HT levels (Gordon & Hen, 2004). A study by Matsuo *et al* (1996), demonstrated that an increase in 5HT within the hippocampus, due to a conflict situation, was associated with anxiety-like behaviour, providing further proof for the relationship between an increase in 5HT and an increase in anxiety-like behaviour.

However, there is an opposing belief in that increases in 5HT are anxiolytic and therefore protect against anxiety (Gordon & Hen, 2004). This view is supported by studies on selective serotonin reuptake inhibitor treatment, which increases 5HT levels by blocking 5HT re-uptake, and decreases anxiety, for example in the suppressed feeding test (Gross & Hen, 2004; Gordon & Hen, 2004). It has been shown that 5HT which is released into the hippocampus also decreases anxiety-like behaviour (Ferguson, et al., 2004; Gordon & Hen, 2004). Synaptotagmin IV (Syt IV) is a secretory vesicle protein that is involved in neurotransmitter release (Ferguson, et al., 2004). In Syt IV knockout mice there was a decrease in anxiety-like behaviour observed in the elevated plus maze and light/dark exploration test. Anxiety-like behaviour has been suggested to be related to the anxiolytic action of 5HT (Ferguson, et al., 2004). A study by Graeff et al (1996) demonstrated that patients suffering from social anxiety disorder, that were treated with D-fenfluramine,

which increased 5HT release, had reduced anxiety when speaking in front of a video camera (Marcin & Nemeroff, 2003).

The 5HT transporter, 5HTT, has also been implicated in anxiety studies (Holmes, et al., 2003a; 2003b; 2003c; Gross & Hen, 2004). It modulates 5HT transmission by re-uptake of 5HT from the synaptic space (Holmes, 2003b; 2003c; 2008). It has been observed in 5HTT polymorphism studies that, the 5-HydroxyTryptamine Transporter Gene-Linked Polymorphic Region (HTTLPR) allele is associated with increased anxiety (Holmes, et al., 2003a). The HTT polymorphism (SLC6A4), which determines 5HTT levels and 5HT re-uptake, leads to decreased 5HTT activity levels and anxiety, due to the increase in 5HT within the forebrain (Holmes, et al., 2003c; 2008). Other studies using mice with a (5HTT $-/-$) mutation, show an increase in 5HT extracellular levels, due to a lack of 5HT reuptake, and an anxiety-like behaviour profile (Holmes, et al., 2003a; 2003c; Gross & Hen, 2004). However, 5HTT is not the only transporter related to anxiety. Studies have demonstrated that mice lacking another 5HT transporter, SERT, have an increase in extracellular 5HT, decrease in tissue 5HT and display anxiety-like behaviour (Patterson, et al., 2005). However, over expression of SERT is associated with a decrease in anxiety-like behaviour (Holmes, 2008). A study by Patterson *et al* (2005) demonstrated that (SERT $-/-$) x (BDNF $+/-$) mice consist of decreased 5HT levels within the brain and increased anxiety-like behaviour (Patterson, et al., 2005), therefore indicating increased 5HT levels are anxiolytic.

Serotonin receptors are also involved in anxiety-like behaviour. 5HT_{1B} knockout mice display less anxiety-like behaviour, but tend to be more active in the open field and elevated plus maze paradigms (Ramboz, et al., 1998). However, it has also been

suggested that 5HT_{1B} knockout mice do not demonstrate any change in anxiety-like behaviour (Holmes, 2008). The 5HT_{1A} receptor subtype seems to be the most popular target for anxiety studies. 5HT_{1A} knockout mice tend to display a decrease in exploratory behaviour but an increase in anxiety-like behaviour (Holmes, 2008) when compared to 5HT_{1B} knockouts (Ramboz, et al., 1998). It has been suggested that an increase in 5HT and anxiety-like behaviour might be a result of the absent 5HT_{1A} autoreceptor (Ramboz, et al., 1998). However, studies have also shown that only when deleting 5HT_{1A} receptors in postnatal weeks will there be an increase in anxiety-like behaviour in adulthood. When deleting the same receptor in adulthood, there will be no change in the anxiety-like behaviour (Holmes, 2008).

5HT activity has also been investigated in human studies. It is believed that 5HT is involved in social anxiety disorder (Marcin & Nemeroff, 2003). In patients suffering from generalized anxiety disorder, a decreased level of 5HT cerebro spinal fluid is observed (Connor & Davidson, 1998). Panic disorder patients on the other hand tend to present with an abnormality in platelet 5HT uptake (Cameron & Nesse, 1988).

1.4.2 Noradrenaline

The noradrenergic system has many functions within the brain, some of which are: arousal in response to a threatening situation, involvement in fear responses within the amygdala and retrieval of fear memory (Marcin & Nemeroff, 2003; Harvey, et al., 2006). The function of NA in psychological disorders has been extensively discussed, as NA reuptake

inhibitors, such as imipramine (Ferguson, et al., 2004), are extensively used for the treatment of some of these disorders (Kim & Gorman, 2005; Kobayashi, et al., 2008).

During times of stress and anxiety, NA has been found to be increased in the locus coeruleus (LC) (Koob, 1999), hippocampus (Cenci, et al., 1992; Koob, 1999), PFC (Finlay, et al., 1994) and the medial prefrontal cortex (mPFC) (Finlay, et al., 1994) in both rodents (Rossetti, et al., 1990; Finlay, et al., 1994; Vahabzadeh & Fillenz, 1994; Arborelius, et al., 1999; Chen, et al., 2004) and humans (Cameron & Nesse, 1988). During rodent maternal separation (stressor) studies, there is an increase in NA observed within the hypothalamus (Arborelius, et al., 1999). There was also an increase in NA within the dorsal raphe nucleus (DRN), when animals were exposed to adolescent defeat (stressor). The NA increase was associated with the increased time and distance spent within the open arms of the elevated plus maze which suggests less of an anxiety-like behaviour profile (Blumberg, et al., 2009). Foot shock (stressor) increases NA levels in the PFC, which are reduced by the use of diazepam (Finlay, et al., 1994). Acute tail pressure (stressor) which is anxiogenic, also increases NA levels in the mPFC (Finlay, et al., 1994). However, the NA response was greater when rats had been previously exposed to chronic cold (Finlay, et al., 1994). Another form of stress used in animal studies is immobilization stress (Cenci, et al., 1992). Here, rodents are placed in an enclosed plastic frame with a flexible wire mesh sheet (Zhang, et al., 1995). Rodents that have undergone immobilization stress, mild handling or tail pinch stress tend to display increased levels of NA in the hippocampus (Cenci, et al., 1992).

NA seems to function together with corticotropin releasing factor (CRF) during stress and anxiety (Arborelius, et al., 1999; Marcin & Nemeroff, 2003). There are synaptic connections between CRF terminals and NA dendrites in the LC (Arborelius, et al., 1999; Sullivan, et al., 1999; Kagamiishi, et al., 2003; Kim & Gorman, 2005), so that when CRF is administered into the LC, a corresponding increase in NA release is observed (Koob, 1999; Marcin & Nemeroff, 2003). CRF administration is also associated with anxiety-like behaviour portrayed in the open field and the light/dark test (Arborelius, et al., 1999). There is also an anxiolytic effect observed when CRF receptor antagonists are used, which may be functioning by inhibiting NA and 5HT (Kagamiishi, et al., 2003). It has previously been suggested that anxiolytic drugs, such as diazepam, inhibit neuronal activity and NA turnover within the LC (Rossetti, et al., 1990).

There is also evidence implicating adrenoreceptors in anxiety (Zhang, et al., 1995). A_2 receptor agonists, such as clonidine, which reduce NA activity, produce anxiolytic effects. While α_2 -adrenoreceptors antagonists such as, yohimbine which stimulate NA release, produce anxiogenic effects (Clement & Chapouthier, 1997; Connor & Davidson, 1998; Sullivan, et al., 1999). However on the other hand the β -adrenergic antagonist, propranolol, is used as an anxiolytic and inhibits memory consolidation (Cameron & Nesse, 1988; Debiec & Ledoux, 2004).

Patients suffering from social anxiety disorder display NA system abnormalities (Marcin & Nemeroff, 2003). In generalized anxiety disorder, patients tend to have a reduced number of α_2 adrenergic receptor binding sites found on platelets (Cameron & Nesse, 1988; Connor & Davidson, 1998). An increase in central NA levels in patients with post traumatic stress

disorder is associated with a rise in peripheral sympathetic nervous system arousal in these same patients (Connor & Davidson, 1998). There is also an increase in α_2 -adrenoreceptor sensitivity in patients suffering from panic disorder (Connor & Davidson, 1998), although in panic disorder, central NA secretion is unaltered (Connor & Davidson, 1988).

1.4.3 Dopamine

DA is the main neurotransmitter responsible for motivated action (Pani, 2000) and functions in the regulation of emotion, along with its association with stress responses (Cenci, et al., 1992; Dazzi, et al., 2001; Harvey, et al., 2006). DA is involved in anxiety disorders and the dysregulation of the DA system may be responsible in part for social anxiety disorder (Marcin & Nemeroff, 2003). Studies have indicated that there was an increase in DA concentration in the rat PFC during times of acute stress, when tail pressure was applied and the increased DA levels were reduced when benzodiazepines, specifically diazepam, were used to treat the anxiety-like behaviour (Finlay, et al., 1994; Morrow, et al., 1999; Dazzi, et al., 2001). Acute stress seems to increase DA levels within the PFC more than any other brain region (Dazzi, et al., 2001). Other stresses, such as immobilization stress, tend to cause a rise in DA levels within the striatum (Zhang, et al., 1995), while mild stress such as handling, increases DA in the mPFC (Cenci, et al., 1992). Pawlak *et al* (2000), demonstrated increases in DA levels in the hippocampus and striatum too, after water emersion (stressor) and restraint (stressor). However, the DA levels returned to normal, after one to two days after the stress was applied (Pawlak, et al., 2000). Mallo *et al* (2007), suggested that cocaine administration and tail pinch tests (stressor), also increased DA levels, but in the nucleus accumbens (NAc) of high motor activity rats moving in a circular corridor, and not in low motor activity rats. Another study conducted

on the NAc demonstrated that loose restraint (stressor) increased DA levels within the NAc, but these levels returned to normal if stress was applied chronically (Pani, et al., 2000). Similar to NA, the DA levels in the mPFC of rats were expected to be increased (Pani, et al., 2000). Stress exposure has also been implied to impair rodent working memory (Dazzi, et al., 2000).

It is generally believed that once stressful events occur, it is likely that anxiety may follow (Dazzi, et al., 2001). The activation of the mesocortical DA region by stress is a likely candidate for the pathophysiology of mood disorders such as anxiety (Dazzi, et al., 2001; Harvey, et al., 2006). An over activation of the dopaminergic pathway may cause an increased fear response, while an inhibition of this pathway may cause a decrease in conditioned fear (Harvey, et al., 2006). D₁ and D₂ receptor agonists, seem to display anxiogenic-like effects (Simon, et al., 1993). D₁ antagonists have been shown to have an anxiolytic effect on rats in the light/dark test, as the rats had a decreased latency in entering the white compartment (Simon, et al., 1993). It is possible that D₁ receptor antagonists reduce anxiety like behaviour in rats, by decreasing DA levels (Simon, et al., 1993).

However, the D₁ and D₂ receptors are not the only subtypes to be investigated in anxiety studies. In a study by Dulawa *et al* (1999), mice that lacked D₄ receptors made fewer entries into the centre of the open field therefore displaying an anxiogenic behaviour. These mice also tended to display reduced exploration of the open field. Therefore the D₄ receptor seems to be involved in the regulation of novelty-seeking behaviour (Dulawa, et al., 1999). While, D₄ receptor deficient mice display a decrease in locomotor activity in the open field, D₃ receptor deficient mice display an increase in locomotor activity (Steiner, et

al., 1997). D₃ receptor deficient mice also seem to have anxiolytic behaviour as they entered the centre of the open field more often than the control rats and they also spend more time on the open arms of the EPM (Steiner, et al., 1997).

Post traumatic stress disorder postmortem patients, who were previously combat veterans, indicated that the Taq1A variant of the DRD₂ gene of the D₂ receptor may play a role in the severity of the anxiety suffered by these patients (Lawford, et al., 2006). The variant gene is associated with differences in D₂ receptor density (Lawford, et al., 2006). The A1 + allelic status may be involved in the general ability of a person to suffer from anxiety and depression.

1.4.4 GABA and Glutamate

GABA is the main inhibitory neurotransmitter (Mombereau, et al., 2004; Wu, et al., 2007), while glutamate is the main excitatory neurotransmitter in the brain (Braga, et al., 2003). A balance between excitation and inhibition in the brain is important for normal brain function (DuBois, et al., 2006; Wu, et al., 2007). However hyperexcitation or reduced inhibition may cause anxiety-like behaviour (Wu, et al., 2007). Benzodiazepines (BZD), which target GABA_A receptors, are regularly prescribed for anxiety disorders (Holmes, et al., 2003; Wu, et al., 2007) and function by increasing the GABA concentration within the brain and therefore inducing an inhibitory effect on the central nervous system (Petersen & Jensen, 1984; Quintero, et al., 1985; Gross & Hen, 2004; Kim & Gorman, 2005).

Patients suffering from panic disorder tend to display reduced GABA levels in the occipital cortex (Kim & Gorman, 2005; Wu, et al., 2007) and hippocampus (Crestani, et al., 1999). However, generalized anxiety disorder patients may have a genetic mutation in the GABA receptor which causes a decrease in receptor binding and therefore the anxiety (Kim & Gorman, 2005). The patients also tend to have reduced peripheral GABA_{B2} binding sites on platelets and lymphocytes, along with a decreased sensitivity on GABA_{B2} receptor subunits in the brain (Connor & Davidson, 1998).

GABA receptor dysfunction has been implicated in the development of anxiety disorders (Kosel, et al., 2004; Wu, et al., 2007). The two different GABA receptors found within the brain are the ionotropic GABA_A receptor and the metabotropic GABA_B receptor (Mombereau, et al., 2004a; Wu, et al., 2007). Direct activation of GABA_A receptors causes a decrease in anxiety-like behaviour in rodents (DuBois, et al., 2006). GABA_A receptors consist of 17 subunits, but the main being the γ^2 subunit (Belzung & Griebel, 2001; Chandra, et al., 2005), which is important for GABA_A receptor clustering and for BZD binding (Crestani, et al., 1999; Chandra, et al., 2005). Studies have shown that γ^2 knockout mice have an increased anxiety-like behaviour which is depicted by a decrease in open arm entries in the EPM and a decrease in locomotor activity in the open field (Chandra, et al., 2005). It may therefore be suggested that GABA_A receptors with the γ^2 subunit may be involved in anxiety (Hodge, et al., 2002; Chandra, et al., 2005). Other studies however, have shown that GABA_A receptors that contain the α_2 subunit are involved in the anxiolytic effects of benzodiazepines (Gross & Hen, 2004). A study by Dias *et al* (2005), demonstrated that the α_3 agonist, TP003, produced an anxiolytic effect in the EPM, therefore GABA_A α_3 receptor subunits may be involved in the regulation of anxiety (Dias, et al., 2005). Studies on the B₂ subunit of the GABA_A complex have demonstrated the

anxiolytic action of B₂ agonists and the anxiogenic action of B₂ inverse agonists (Clement & Chapouthier, 1997). It has been suggested that B₂ receptor secretion of inverse agonists (BZD receptor ligands which modulate GABA function in the opposite direction to the BZD agonists), decreases GABAergic tone and in turn causes anxiety-like behaviour or stress in animals (Petersen & Jensen, 1984; Clement & Chapouthier, 1997). And so, B₂ GABA_A receptor subunit dysfunction may be involved in anxiety disorders (Clement & Chapouthier, 1997). On the other hand, the other type of GABA receptor, the GABA_B receptor, has also been shown to be involved in anxiety. Deletion of the GABA_{B1} subunit produces an increased anxiety-like behaviour in the light/dark box (Mombereau, et al., 2004). However, classic benzodiazepines are not effective in decreasing the anxiety-like behaviour in GABA_{B1} mice (Mombereau, et al., 2004).

Glutamate has also been implicated in the modulation of anxiety. For example, the administration of a glutamate antagonist into the amygdala blocked fear-potentiated startle responses in rats (Lang, et al., 2000). While during stressful conditions, there is an increase in glutamatergic activity in the PFC (Kim & Gorman, 2005). Glutamate transmission through NMDA receptor activation has also been implicated in anxiety and fear conditioning (Harvey, et al., 2004; Bessiere, et al., 2007). Anxiety knockout studies have been conducted on the glutamate receptor mGluR7 and galanin GAL-R₁ receptor and both receptors play a role in anxiety and depression (Cryan & Holmes, 2005). Glutamate influences inhibitory transmission through kainate (KA) receptors. KA receptors regulate the release of glutamate and GABA (Braga, et al., 2003). These receptors include GluR5, R6, R7, KA₁ and KA₂ (Wu, et al., 2007). For example GluR5 ^{-/-} mice display an increased anxiety-like behaviour profile which is believed to be caused by inhibited GABAergic transmission. When a GluR5 agonist, is administered in mice, there seems to be a decrease

in anxiety-like behaviour, while, a GluR5 antagonist, causes an anxiogenic profile in mice (Wu, et al., 2007).

1.4.5 Corticosterone

Glucocorticoid receptors, which include the mineralocorticoid receptor (MR, type I) and the glucocorticoid receptor (GR, type II), are found in the brain. The GR receptors become occupied during a stressful event when cortisol (in the human) or CORT (Walker, et al., 2004) (in the rat) is released from the adrenal cortex (Arborelius, et al., 1999). CORT is believed to be responsible for the suppression of the hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis during acute stress, by a negative feedback mechanism on the HPA axis, which in turn inhibits further secretion of glucocorticoids (Connor & Davidson, 1998; Arborelius, et al., 1999; Grillon, et al., 2007). In an acute stressor study in rats, it was found that immediately after tail pinch (stressor), plasma CORT levels were increased, but 30 minutes later this increase was not as elevated (Kirby, et al., 1997b). Once the stress response is completed, the glucocorticoid levels decrease, GR density increases and the feedback mechanism is again normalised (Connor & Davidson, 1998). Rats exposed to foot shock (acute stressor) one or three times a day display an increase in CORT, when a stressor is applied ten days later because of the lack of suppression of the HPA axis by glucocorticoids (Chotiawat & Harris, 2006). However in chronic stress, the levels of cortisol are less than those during acute stress (Connor & Davidson, 2006). Another form of stress is seen when rats, which are social animals, are separated either from their mothers or partners (Connor & Davidson, 1998; Kalinichev, et al., 2002; Bosch, et al., 2009). In rats which have been previously exposed to maternal deprivation stress or *in*

utero stress, show increased levels of CORT in response to behavioural applied stressors in adulthood. This is believed to be an exaggerated glucocorticoid response experienced in adulthood (Connor & Davidson, 1998). Also, an increase in GR type II is expected when rat pups are handled by humans in the first weeks of life (Connor & Davidson, 1998). The stress of being maternally separated is also seen in the first two weeks of life (Kalinichev, et al., 2002). In Long-Evans rats, an exaggerated secretion of CORT is seen once a stressor is applied after three hours of maternal separation. This stress was accompanied by anxiety-like behaviour (Kalinichev, et al., 2002). In male rats separated from their female partners, a higher CORT concentration is also observed, compared to control rats (Bosch, et al., 2009).

The HPA axis has been implicated in the modulation of anxiety (Shepard, et al., 2000). Anxiety is increased by an elevation of CORT while a decrease in CORT release is associated with a decrease in anxiety, which has been induced by a stressor (Shepard, et al., 2000; Barbier & Wang, 2009). Studies in rats have shown that CORT implants in the amygdala are related to a decrease in open arm preferences in the elevated plus maze, which suggests an anxiety-like behaviour profile (Shepard, et al., 2000). An acute stressor may also induce behavioural changes such as anxiety in animals. For example, acute restraint stress is associated with increased anxiety-like behaviour as shown by the elevated plus maze and light/dark box (Chotiawat & Harris, 2006). Following 21 days after inescapable foot shock was applied to rats, these rats displayed increased anxiety-like behaviour within the open field, showing that exposure to an acute stressor may result in sustained anxiety-like behaviour (Chotiawat & Harris, 2006). Behavioural tests themselves have also been suggested to be stressful. Chotiawat & Harris (2006), showed that re-restrained (RR) mice had an increased in CORT levels after being exposed to a variety of

behavioural tests (Chotiawat & Harris, 2006). Landgraf *et al*, (2002) have shown that high anxiety behaviour (HAB) mice tend to have larger levels of CORT than low anxiety behaviour (LAB) mice (Landgraf & Wigger, 2002). HAB mice seem to have a hyperactive HPA axis which is also seen in cases with psychiatric patients (Landgraf & Wigger, 2002).

Patients that suffer from anxiety disorders display variability in the concentration of cortisol (Cameron & Nesse, 1988). In phobia patients the concentration of cortisol is increased, in General Anxiety Disorder patients the urinary cortisol concentrations may be elevated or normal and in Panic Disorder patients the cortisol blood levels are normal or slightly elevated (Cameron & Nesse, 1988). However, on the other hand, PTSD patients have an insufficient level of cortisol in response to a stressful event and this is believed to inhibit stress induced responses such as anxiety (Connor & Davidson, 1998; Harvey, et al., 2006). Maltreated children suffering from post traumatic stress disorder, have larger amounts of urinary cortisol in comparison to control children (Connor & Davidson, 1998).

1.5 Neurotransmitters and their association with pain

1.5.1 Serotonin

The involvement of 5HT in studies of hyperalgesia has been well documented (Tenen, 1967; Telner, et al., 1977). It is believed that after tissue injury, 5HT is released from platelets and activates nociceptors (Bannister, et al., 2009). 5HT also causes the release of substance P, calcitonin, gene-related peptide and neurokinin-A from afferent fibres

(Bannister, et al., 2009). Previous studies demonstrated that lowering 5HT in turn caused an increase in pain sensitivity in rats (Tenen, et al., 1967). Telner *et al.* (1977) demonstrated that increasing the level of 5HT within the brain of rats caused hypoalgesia in rats when exposed to inescapable shock. However, when 5HT levels within the brain were decreased by dorsal raphe lesions, rats displayed hyperalgesia (Telner, et al., 1977). In human studies, injecting low concentrations of 5HT intradermally into the masseter muscle of the jaw causes hyperalgesia (Bardin, 2011). Whereas, a decrease in mechanical hyperalgesia is seen when exogenous 5HT is delivered to the spinal cord (Bannister, et al., 2009). In addition, it is believed that 5HT, released from brainstem neurons onto inhibitory 5HT receptors in the spinal cord, is needed for RVM-mediated morphine analgesia (Bannister, et al., 2009).

5HT receptors have also been implicated in hyperalgesia studies in the rat. Hyperalgesia may occur when rats are injected intradermally with a 5HT_{1A} agonist (Sommer, 2004). On the other hand, some scientists believe that brainstem 5HT has antinociceptive qualities that are mediated by 5HT_{1A} receptors (Bannister, et al., 2009). However, 5HT in the brainstem has been suggested to be pronociceptive through 5HT_{2A} and 5HT₃ receptors (Bannister, et al., 2009). Treatment with a 5HT_{2A} antagonist preceded by formalin injection, causes a decrease in the rats' pain response, suggesting the involvement of 5HT in peripheral sensitization in these rats (Abbott et al., 1997). Therefore it is reasonable to conclude that 5HT contributes to peripheral sensitization in injured or inflamed tissues. On the other hand, others have suggested that 5HT may not be responsible for inducing pain itself, but instead just enhances the effects of other mediators found in inflamed tissues, such as bradykinin, PGE₂ and histamine (Sommer, 2004).

1.5.2 Noradrenaline

NA is involved in the pain inhibitory system by inhibiting nociceptive transmission (Jasmin, et al., 2002). It is also involved in hyperalgesia. Studies have demonstrated that NA can increase thresholds and latency to noxious stimuli when α_2 -adrenoreceptors in the brain and spinal cord have been stimulated (Jasmin, et al., 2002). Hyperalgesia is also achieved in rats by acute lesions or inhibition of NA within afferent neurons of the spinal cord (Sagen & Proudfit, 1984). Moreover, others have suggested that a continuous stimulation of α_2 -adrenoreceptors is needed if mice are to remain with a normal nociceptive threshold (Jones, 1991). It has also been suggested that NA does not act alone but if this neurotransmitter is released chronically, it may cause the release of substance P chronically too and this in turn could decrease nociceptive thresholds stimulated by thermal cues (Jasmin, et al., 2002). In accordance with the studies above, Mochizucki (2004) suggested that an increase in 5HT locally and an increase in NA transmission may lead to antinociceptive effects stemming from the descending inhibitory pain pathways in the central nervous system (Mochizucki, 2004).

1.5.3 Dopamine

DA is believed to be involved in the modulation of pain perception and analgesia originating naturally from the brain and spinal cord (Gao, et al., 2010). Analgesia is achieved with the activation of D₂ receptors, while activation of D₁ receptors within the periaqueductal gray, attenuates pain (Gao, et al., 2010). However, others have demonstrated that stimulation of D₁ receptors within the spinal cord and cerebral cortex may lead to an increase in nociception, while increased D₂ receptor activity within the

spinal cord reduces nociception (Wood, 2008). Flores et al (2004), demonstrated that injecting 6-hydroxydopamine into the ventro-lateral periaqueductal gray to remove dopaminergic input, results in the attenuation of antinociceptive effects of administered morphine (Flores, et al., 2004; Meyer, et al., 2009). Others have suggested that DA reuptake inhibition in the rostral agranular insular cortex resulting in analgesia, stems from descending inhibition (Wood, 2008). A study demonstrated that injecting a DA reuptake inhibitor into the rostral agranular insular cortex, results in increased pain thresholds to thermal stimuli and also achieves analgesia for pain produced from administered formalin (Burkey, et al., 1999).

1.5.4 Corticosterone

The link between stress and pain sensitivity has been investigated immensely over the years. Stressors such as repeated sound stress (Hata, et al., 1988) and water avoidance stress (Schwetz, et al., 2003; Khasar, et al., 2009), to name a few, have been shown to decrease rat nociceptive thresholds. Researchers have suggested that children that are stressed grow up to suffer from increased pain sensitivity (Davis, et al., 2005). In addition, it is believed that stress in early life is correlated with chronic pain syndromes such as fibromyalgia (Davis, et al., 2005; Khasar, et al., 2008). Reasonably, HPA axis has therefore been implicated in chronic pain syndromes (Khasar et al., 2008). In a study conducted on rats, it was demonstrated that when rat pups were exposed to neonatal limited bedding (stressor), as adults the same rats experienced mechanical hyperalgesia within the muscle but not the skin (Green, et al., 2011). Moreover, these rats demonstrated an increased basal CORT concentration along with increased responses to stress (Green, et al., 2011). This led researchers to suggest that adult rats suffering from chronic stress have

enhanced inflammatory mediated induced hyperalgesia (Green, et al., 2011). In another study, investigating stress and hyperalgesia, Khasar et al (2008) demonstrated that stress induced mechanical hyperalgesia is mediated by CORT and epinephrine, which function by changing primary afferent nociceptor pathways (Khasar, et al., 2008).

Specifically when CORT is administered into the amygdala of rats, decreased visceral and somatic pain thresholds result, along with anxiety-like behaviour (Myers, et al., 2007). In a study by Hong et al (2011), it was demonstrated that rats that are stressed by water avoidance stress tend to display visceral hyperalgesia and the authors believe that the HPA axis and in turn CORT is responsible for the decreased thresholds (Hong, et al., 2011).

1.5.5 GABA and Glutamate

Metabotropic glutamate receptors (mGluRs) and metabotropic GABA receptors (GABA-B) are believed to regulate nociceptive transmission and pain (Goudet, et al., 2009).

Studies using GABA, conducted on the rostral agranular insular cortex (RAIC), demonstrated that increasing GABA concentration locally results in analgesia arising from stimulation of descending inhibitory spinal nociceptor neurons (Jasmin et al., 2003). In addition, stimulating GABA-B receptors in the same region results in thermal hyperalgesia tested using the heat paw withdrawal test (Jasmin, et al., 2003). However, GABA concentration also affects mechanical hyperalgesia. A study conducted by, Goudet et al (2009), demonstrated that GABA-B₁ knockout mice also display decreased withdrawal thresholds tested using the tail flick test (mechanical hyperalgesia) (Goudet, et al., 2009).

Previous studies have suggested that injection of L-glutamate or other glutamate receptor agonists into the hindpaw of rats result in thermal and mechanical hyperalgesia (Jin, et al., 2011). Capsaicin injection also leads to the development and maintenance of thermal hyperalgesia, believed to involve GluRs found in peripheral afferent fibres (Jin, et al., 2011). Meller et al (1993) demonstrated that the activation of mGluRs led to a decrease in withdrawal thresholds, tested using the tail flick test (mechanical hyperalgesia). Therefore, glutamate receptors are involved in both thermal and mechanical hyperalgesia. In human studies along with animal studies, it has also been shown that when injecting glutamate into muscle tissue, mechanical sensitivity results (Cairns, et al., 2003). In a study comparing pain induced by glutamate and capsaicin it was found that the duration of glutamate pain was longer than capsaicin, although nociceptive activity was greater in capsaicin induced pain (Gibson, et al., 2009). Therefore glutamate may be greatly involved in the maintenance of pain.

1.6 Aims of the dissertation

I specifically investigated whether anxiety-like behaviour (measured using the open field paradigm) develops in rats postoperatively and whether the duration of this anxiety-like behaviour after surgery correlates with the duration of postoperative hypernociception (measured using standard behavioural nociceptive testing techniques). I then investigated whether the behavioural responses of the animals after surgery were associated with changes in biological markers of anxiety, such as brain 5HT, NA, DA, GABA and glutamate levels and plasma CORT levels.

Animal models of human pain conditions which assess higher order nociceptive processing are important for the long-term utility of the model in pain research. The pain experience in humans consists of sensory, affective and cognitive components and holistic treatment of pain addresses all three components (Melzack & Casey, 1968). Unfortunately, most current animal models of postoperative pain only assess the effect treatments have on the sensory component of pain, without addressing the affective and cognitive components of pain. I therefore aimed to study the affective component of pain by assessing anxiety-like behaviour in the postoperative animal model of hypernociception, just as anxiety-like behaviour has been successfully studied in the neuropathic animal model of pain.

Chapter 2

Methods

2.1. Experimental animals and housing

Two-hundred adult male Sprague-Dawley rats, bred at the National Health Laboratory Service (NHLS) and weighing 200-300g at the start of surgery, were housed individually in cages in a temperature-controlled room (~ 22°C) on a 12 h light : 12 h dark cycle with lights on at 07:00. Food and water were available *ad libitum*. Cages were cleaned twice a week by the staff of the Central Animal Service of the University of the Witwatersrand.

All experiments were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (Clearance numbers AESC 2010/20/04 and AESC 2011/12/04) and experimental procedures were performed in accordance with the principles and procedures described in the University of the Witwatersrand's guide for the care and use of laboratory animals.

2.2 Experimental procedures

2.2.1 Surgery

Rats were anaesthetised with an intramuscular injection of 100mg/kg ketamine (Bayer, South Africa) and 5mg/kg xylazine (Bayer, South Africa). Thereafter, the anaesthesia was maintained with 1-3% inhaled isoflurane (Safeline Pharmaceuticals (PTY) LTD, South Africa), which was delivered to each rat through a nose cone. All rats' abdomens were shaved on the lower left quadrant, exposing the ribs and the area immediately under the

lowest rib. The shaved area was cleaned with chlorhexidine gluconate (Hibitane, AstraZeneca, South Africa).

For rats that underwent surgery (**surgery group**), a 2cm incision was made below and parallel to the lowest rib on the left side of the animal, through the skin, muscle and into the peritoneal cavity. Thereafter a finger was inserted into the abdominal cavity to stretch the wound. The wound was sutured with 4/0 nylon (SRL, Tyco Healthcare, South Africa) and treated with a topical antiseptic (Necrospray, Centaur Labs, South Africa). Each surgery lasted approximately 10 minutes per rat.

No surgical incision was made in the rats that underwent sham surgery (**sham surgery group**). However, a black permanent marker was used to mark the area on the abdomen where surgery was performed on the rats in the **surgery** group, so as to orientate the experimenter, when conducting nociceptive testing (Experiment 1).

2.2.2 Nociceptive behaviour testing (Experiment 1)

Animals were placed on a wire grid (grid bars were 1cm apart while the bars were 0.2cm wide) and under a clear Perspex lid (20 cm x 20 cm x 20 cm) to limit their movement on the grid, but still allow them to respond freely to the nociceptive testing (Figure 1).

Nociceptive testing was performed using an electronic von Frey anesthesiometer (IITC Life Science Instruments, USA) and involved applying a punctuate stimulus to the area immediately adjacent to the wound. The force applied was increased steadily until the

animal withdrew from the stimulus. The force at which each animal withdrew from the stimulus was recorded. An average of six measurements, measured at one minute intervals was taken as an animal's nociceptive threshold on each day of testing.



Figure 1. Nociceptive testing being conducted on a rat resting on the mesh floor under a clear perspex lid, using a von Frey anaesthesiometer. The probe of the anaesthesiometer (mechanical force) is applied between the mesh wire and directly adjacent onto the area where the incision was made on the rats' abdomen.

2.2.3 Anxiety-like behaviour testing (Experiment 2)

The open field paradigm was used to test anxiety-like behaviour in rats that had undergone surgery and sham surgery. The open field paradigm apparatus consisted of a 1 m x 1 m x 0.3 m white Perspex® arena, with a defined, grey, inner zone of 0.4 m x 0.4 m (Figure 2). The apparatus was placed in a room illuminated at (200 lux). Each rat was removed from its cage and placed in the centre of the open field arena and allowed to explore the novel environment for 15 minutes while the activity was captured on video camera. The rat was then returned to its cage. Between tests the apparatus was cleaned with F10SC veterinary disinfectant (Health and Hygiene PTY Ltd, South Africa) and paper towels to eliminate possible olfactory cues. The video camera (Canon digital video camcorder, model DM-MV550i E) was mounted on the ceiling above the apparatus and recorded each rat's movement within the arena. The camera was connected to a computer which tracked and analysed the rat's movements, using ANY-maze software (Stoelting Co., IL, USA). The software calculated movement indices within the inner zone and outer zone, including the total distance moved, time spent within each zone, the number of entries into each zone and the average speed within each zone. I also measured the number of times a rat reared in the open field. The anxiety-like behaviour tests were conducted between 11:00 and 13:00.

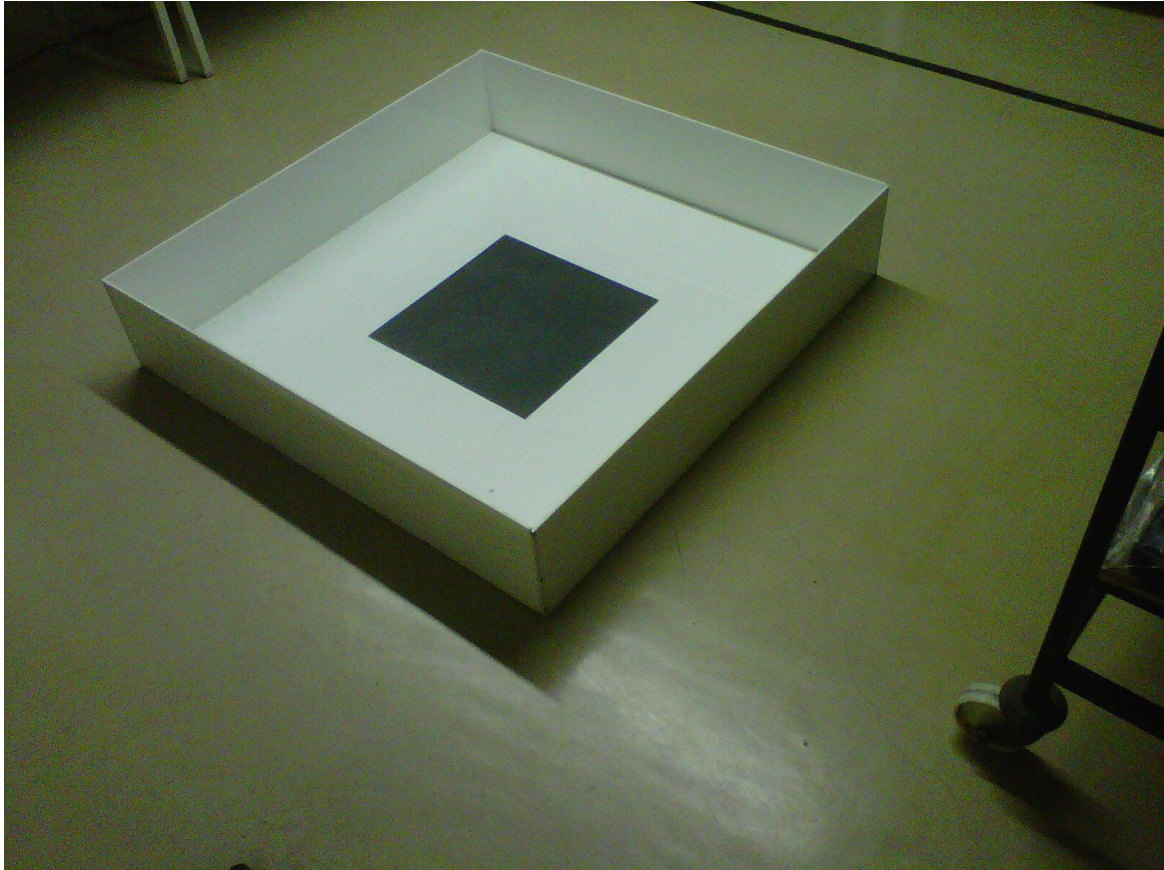


Figure 2. The open field paradigm, made of white and grey perspex, used to test anxiety-like behaviour in rats.

2.2.4 Corticosterone, monoamine, GABA and glutamate analysis

2.2.4.1 Corticosterone analysis

CORT analysis was conducted by me at the medical campus of the University of the Witwatersrand. Rat plasma CORT was analysed in duplicate using a commercially available Radioimmunoassay (RIA) kit (DRG diagnostics, USA). All plasma samples were first diluted with steroid diluent (1:200 dilution). 0.3ml of steroid diluent was then added to tubes one and two, the non-specific binding (NSB) tubes and 0.1ml of steroid diluent was added to tubes three and four, the standard 0 ng/ml tubes. 0.1ml of each of the CORT standards (25, 50, 100, 250, 500 and 1 000) was added to tubes five to 16. 0.1ml of Control 1 (high CORT concentration) was added to tube 17 and 0.1ml of Control 2 (Low corticosterone concentration) was added to tube 18. Then 0.1ml of the 1:200 diluted plasma samples was added to tube 19 through to the last tube in the series. 0.2ml of radioactive corticosterone⁻¹²⁵ was added to all the tubes while anticorticosterone (rabbit gamma globulins) was added to all tubes except tube one and two. After which all tubes were vortexed and incubated at 22 - 25°C for two hours. Following the incubation period, 0.5ml of precipitant solution was added to all tubes. The tubes were then vortexed and placed in a centrifuge at 2 500 rpm for 15 minutes at 4 °C. The supernatant was decanted and the radioactivity of the precipitate counted in the gamma counter. The values presented by the gamma counter were then averaged for all duplicated tubes. The averaged NSB (blank) counts were then subtracted from the averages obtained. These were then the corrected values. The corrected values were divided by the corrected zero calibrator value in order to obtain the percent bound.

2.2.4.2 Monoamine, GABA and glutamate analysis

Brain monoamines (5HT, DA, NA), GABA and glutamate were analysed using Agilent 1200 high performance liquid chromatography (HPLC), which was equipped with an isocratic pump and a autosampler coupled to an ESA Coulochem III Electrochemical detector (with Coulometric flow cell). Data were analysed using Chromeleon® Chromatography management system version 6.8 software.

Please note the process for monoamines, GABA and glutamate analysis is similar for most parts, however there are subtle differences between GABA and glutamate analysis and monoamine analysis, which will be noted in the following text. Monoamines, GABA and glutamate were analysed by the laboratory technician at North West University's pharmacology department and I.

Brain samples were removed from the -80° C freezer, weighed, thawed and 0.5ml of Solution A (for monoamines) and 1ml of solution A (for GABA and glutamate), prepared by dissolving 0.09505g sodium metabisulphite and 0.111672g Na₂EDTA in 800ml distilled water, adding 10.87ml perchloric acid and making up the solution to 1 000ml, was added to each tube. Based on the preferred method of cell disruption for small samples and volume size (Keller et al, 1976), the brain tissue within each tube was ruptured using a sonicator for 12 seconds at 14 µl amplitude. All tubes were then placed on ice for 20 minutes in order to allow for the perchlorate precipitation of protein and extraction of monoamines and neurotransmitters to be completed. The samples were centrifuged at 4° C in an ultra centrifuge for 30 minutes at 15 300 rpm and the supernatant fluid was then

removed and placed in separate tubes. The pH of the tissue extract in the new tube was adjusted to 5 (for monoamines) and 9 (for GABA and glutamate) with the addition of 5 μ /ml of 0.1M potassium acetate. An aliquot of 200 μ l of tissue extract was removed with a pipette and placed into another new tube to which 20 μ l of the internal standard, isoprenaline, was then added. The final sample was vortexed for 10 seconds, centrifuged for 5 minutes at 15 300rpm and 10 μ l were injected into the HPLC column. The results were expressed as ng/g wet weight of tissue. All abovementioned concentrations have been calculated from a standard concentration range of 0.1-20 μ g/ml.

2.3 Protocols

Figure 3, depicts the allocation of animals in the three experiments I conducted, namely nociceptive behaviour testing, anxiety-like behaviour testing, as well as tissue stress and anxiety biomarker analysis.

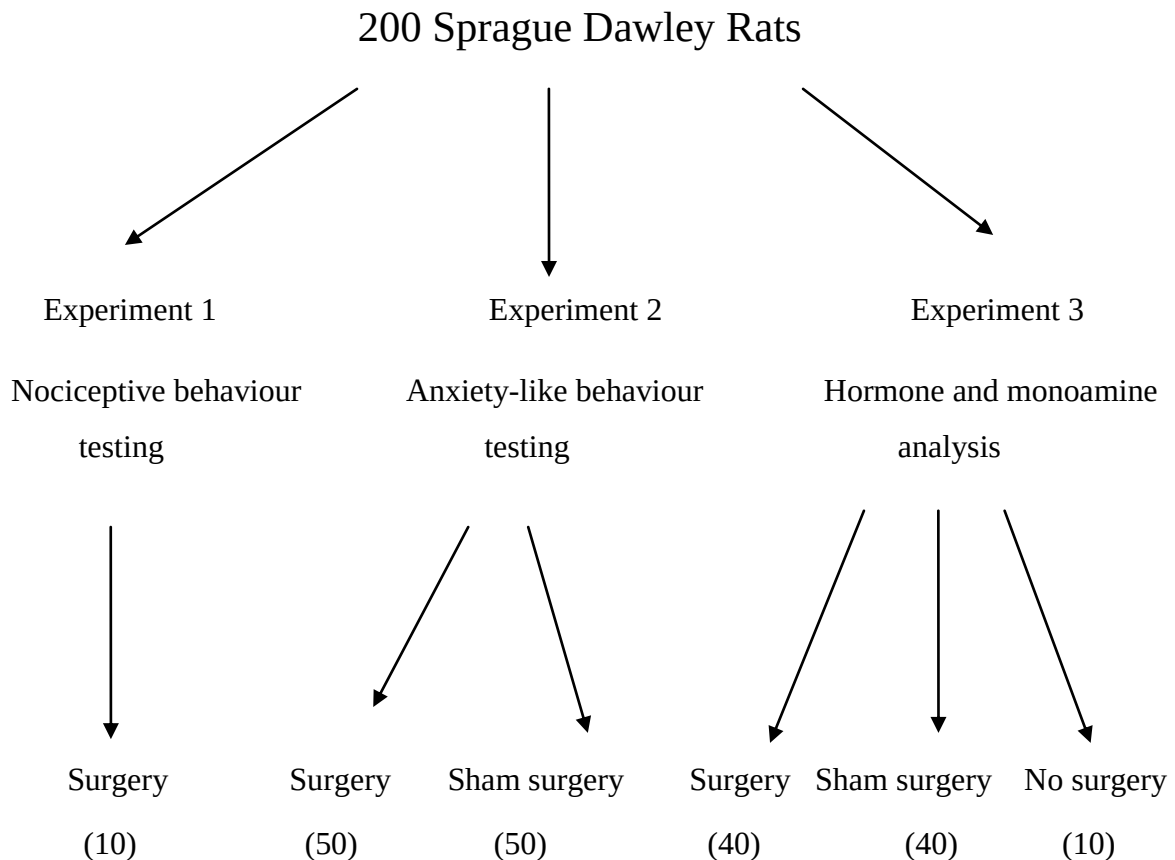


Fig. 3. A diagrammatic representation of the experiments performed in this study and the number of animals used in each experiment. In Experiment 1, baseline testing of nociceptive sensitivity took place four days before surgery. The animals then underwent surgery and nociceptive testing was conducted on day two, three, four, six and nine after surgery. In Experiment 2, animals only underwent anxiety-like behaviour testing once, with ten animals in each group being tested on postoperative day two, three, four, six and nine. In Experiment 3, sample collection was only conducted on each animal once, on one of the following postoperative days: one, two, four and nine. In order to detect a significant difference between postoperative days, at least ten animals need to be placed in each postoperative day group, therefore a large number of animals were used in this study.

2.3.1 Experiment 1: Nociceptive behaviour testing

For 30 minutes per day for three days, ten rats were habituated to the environment in which nociceptive testing would occur. After each habituation session rats were returned to their home cages. Following habituation, baseline nociceptive behaviour testing was conducted daily on all rats, for four days before surgery, using the method described in section 2.2.2. Thresholds of rats were assessed at the intended site of surgery. After surgery, nociceptive testing was conducted on all rats on day two, three, four, six and nine. Testing was always carried out between 11: 00 and 13: 00.

2.3.2 Experiment 2: Anxiety-like behaviour testing

One hundred rats were randomly allocated to either the **surgery** (n = 50) or **sham surgery** (n = 50) group. Ten rats from each of these groups were then tested for anxiety-like behaviour on one of the following postoperative days: two, three, four, six and nine; and were only exposed to the open-field once, to avoid habituation that may alter the anxiety-like behaviour testing results.

2.3.3 Experiment 3: Blood and brain tissue analysis

2.3.3.1 Blood collection

Rats were euthanized with an intramuscular injection of 100mg/kg ketamine (Bayer, South Africa) and 5mg/kg xylazine (Bayer, South Africa) and then decapitated using a guillotine

(Biocom biotech, South Africa). The trunk of the body was tilted into a funnel to facilitate the collection of trunk blood, into a 2ml sterile tube containing EDTA. The tube was then sealed with the lid and placed on ice. The blood was centrifuged at 5000 rpm, at 4°C for 15 minutes to separate the plasma from the rest of the blood contents. Approximately 2ml of plasma was then pipetted out of the test tube using a 1000µl pipette into a 2ml eppendorf tube. All plasma-containing tubes were stored in a -80°C freezer until radioactive immune assays were performed (see section 2.2.4.1).

2.3.3.2 Brain tissue collection

Once the animals had been decapitated on specific days following surgery (see figure 3) and their blood collected, two brain areas, namely the prefrontal cortices and hippocampi of each rat were excised using dissecting apparatus. The left and right prefrontal cortices were dissected separately into a 2ml Eppendorf tube and snap frozen in liquid nitrogen. The left and right hippocampi were also dissected into a 2ml Eppendorf and snap frozen in liquid nitrogen. The tubes were then stored in a -80°C freezer until high performance liquid chromatography was performed on the samples (see section 2.2.4.2).

A total of 90 rats were used for the brain and blood collection described above (see section 2.5.1 and 2.5.2). Forty of these rats underwent surgery and the remaining 40 rats underwent sham surgery. On each of the postoperative days two, three, six and nine, ten rats from the surgery group and ten rats from the sham surgery group were decapitated and their tissues collected. In addition, tissues were also collected from ten age matched rats,

which had no intervention (i.e no surgery or sham surgery). These ten “no intervention” animals represented the control group.

Anxiety-like behaviour testing was conducted on postoperative days two, three, four, six and nine and results showed that rats did not display anxiety-like behaviour on any of the above mentioned postoperative days. It was therefore unnecessary to conduct anxiety biomarker analysis on all the same days. I chose to test the blood and brain biomarker levels on postoperative day one and two, when anxiety would be mostly likely detected, day four which is roughly the middle of the postoperative days and then day nine when hypernociception was no longer observed in rats. Behavioural tests and biomarker tests were not conducted on the same groups of rats so that the open field paradigm did not affect the biomarker levels.

2.4 Data Analysis

Data analysis was performed using software GraphPad Prism version 5.2. Data are presented as mean $\pm$ SD or median IQR. The assessment of nociceptive behaviour after surgery was performed by using a non-parametric Friedman’s test, followed by a Dunn’s post-hoc test. Anxiety-like behaviour data was analysed using a two-way ANOVA with a standard Newman Kewls post hoc test if significant effects or interaction was detected. Monoamine, CORT, GABA and glutamate concentration data, within the brain and blood, were analysed using a Kruskal-Wallis test. A value of $p < 0.05$ was considered as significant for all analyses.

Chapter 3

Results

3.1 Experiment 1: Nociceptive behaviour testing

Surgery was conducted to induce hypernociception in rats (see section 2.2.1). Figure 3.1 shows the withdrawal response threshold measured in ten rats before and after surgery.

For baseline values, each rats' withdrawal response threshold was tested daily for four days before surgery. After surgery, withdrawal response thresholds were tested in each rat on four specific days, namely day two, three, six and nine. A non-parametric Friedman test detected a significant difference in withdrawal response threshold across time (Fig 3.1; $p < 0.001$), with post-hoc Dunn's tests showing a significant decrease in threshold compared to pre-operative levels on days two, three, four and six after surgery. There was no significant difference in response threshold between the pre-operative level and day nine after surgery. Thus, surgery resulted in a reduction in response threshold to a mechanical stimulus, applied immediately adjacent to the wound, for up to six days following surgery.

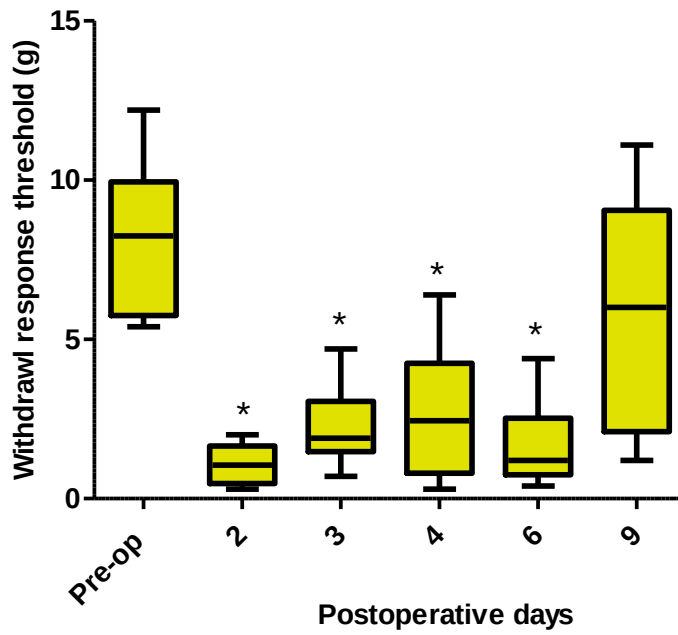


Figure 3.1 Withdrawal response threshold (mean $\pm$ SD) of rats (n=10), before (pre-op) and after surgery. Withdrawal response threshold is expressed in grams. Pre-operative withdrawal response thresholds were averaged for all rats over four days. * $p < 0.05$: postoperative days two, three, four, six vs average of pre-operative days.

3.2 Experiment 2: Anxiety-like behaviour testing

3.2.1 Gross motor activity

Two groups of rats (n=50 per group) underwent surgery or sham surgery (see section 2.2.1) before being tested for postoperative anxiety-like behaviour. Different rats (n=10) in each group were tested on each of the five postoperative days. **Figure 3.2.1** shows the gross motor activity parameters, namely total distance moved, average speed and rearing, for surgery and sham surgery groups of rats, measured using the open field paradigm. A two-way ANOVA detected no significant difference in the total distance moved for rats that underwent surgery and those that underwent sham surgery [Group effect: $F_{(1,90)} = 0.90$, $P = 0.36$] on any of the postoperative days [Time effect: $F_{(4,90)} = 0.90$, $P = 0.49$] and also detected no interaction effect between the two [Interaction effect: $F_{(4,90)} = 2.00$, $P = 0.08$]. Thus, on average the rats, whether exposed to surgery or sham surgery, covered the same distance exploring the open field. A two-way ANOVA also showed no significant difference in the average speed of rats that underwent surgery or those that underwent sham surgery (Fig. 3.2.1) [Group effect: $F_{(1,72)} = 0.40$, $P = 0.52$] on any of the postoperative days [Time effect: $F_{(4,72)} = 0.10$, $P = 0.97$] and also detected no interaction effect between the two [Interaction effect: $F_{(4,72)} = 1.00$, $P = 0.42$]. Thus, the average speed on which rats moved while exploring the open field was not significantly different in rats exposed to surgery compared to those exposed to sham surgery. A two-way ANOVA, and subsequent post-hoc analysis, detected a significant time effect on postoperative day two [Time effect: $F_{(4,90)} = 9.00$, $P < 0.0001$] and a significant interaction effect between time and group [Interaction effect: $F_{(4,90)} = 5$, $P = 0.0006$], in the rearing behaviour of rats that underwent surgery and those that underwent sham surgery. However, no significant group

effect was detected [Group effect: $F_{(1,90)} = 9\text{e-}005$, $P = 1.00$]. The decrease in rearing behaviour of sham surgery rats on postoperative day two, was an anomalous finding.

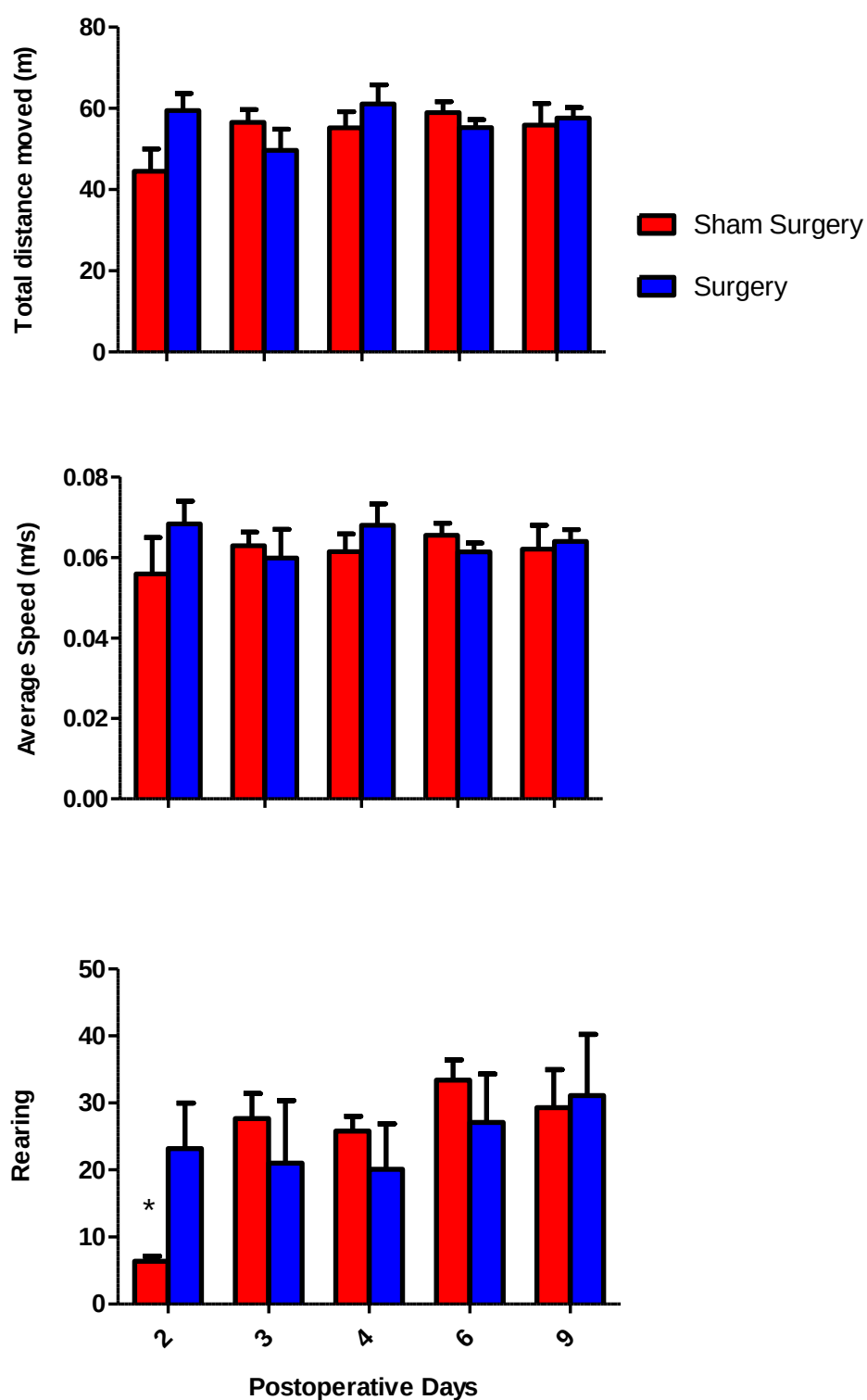


Figure 3.2.1 The total distance moved, the average speed and rearing behaviour (mean $\pm$ SD) measured in rats (n=10 per group per day) in the open field paradigm, as an index of gross motor activity, after rats were exposed to either surgery or sham surgery. * p < 0.05 sham surgery vs surgery rats on postoperative day 2.

3.2.2 Anxiety parameters

Figure 3.2.2 shows measurements of anxiety-like behaviour using the open field paradigm.

A two-way ANOVA showed no significant difference on any of the postoperative days [Time effect: $F_{(4,90)} = 1.00$, $P = 0.23$] in the number of inner zone entries in rats that underwent surgery and those that underwent sham surgery [Group effect: $F_{(1,90)} = 0.004$, $P = 0.95$]. There was also no significant effect in the interaction between time and group [Interaction: $F_{(4,90)} = 2.00$, $P = 0.13$]. Similarly, a two-way ANOVA showed there was no significant difference in the time spent within the inner zone for rats that underwent surgery and those that underwent sham surgery [Group effect: $F_{(1,72)} = 0.20$, $P = 0.68$] on any of the postoperative days [Time effect: $F_{(4,72)} = 3.00$, $P = 0.70$]. There was no significant interaction between the time and group [Interaction effect: $F_{(4,72)} = 2.00$, $P = 0.18$]. A two-way ANOVA also showed that there was no significant difference in the distance moved within the inner zone for rats that had undergone surgery and those that had undergone sham surgery [Group effect: $F_{(1,72)} = 0.20$, $P = 0.66$] on any of the postoperative days [Time effect: $F_{(4,72)} = 2.00$, $P = 0.09$]. There was no significant interaction effect between the two either [Interaction effect: $F_{(4,72)} = 1.00$, $P = 0.24$]. Thus, rats that underwent surgery did not show any signs of anxiety-like behaviour, as assessed by avoidance of open spaces using the open field paradigm.

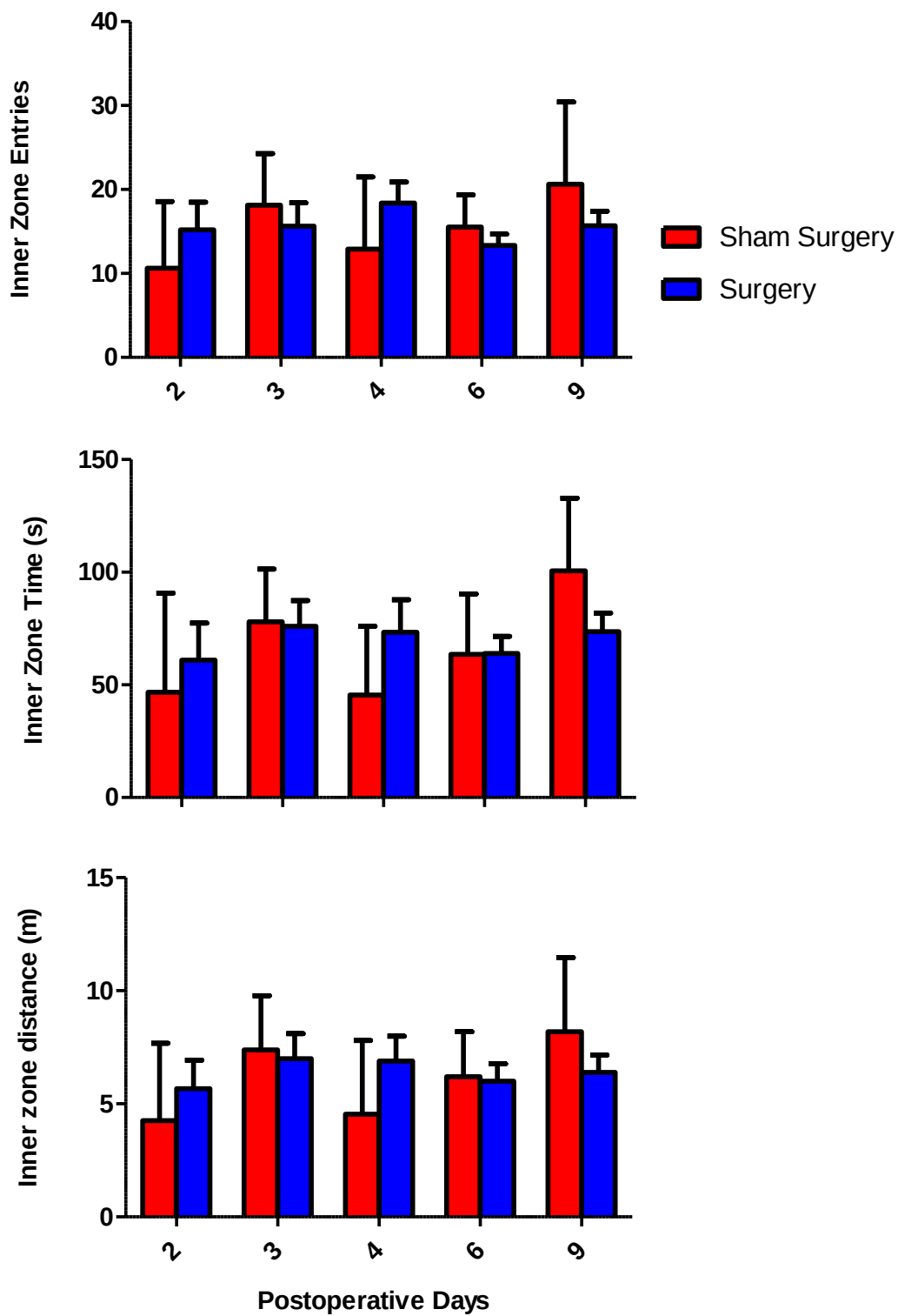


Figure 3.2.2 Indices measured in the inner zone of the open field paradigm namely, number of entries, time spent and distance travelled (mean $\pm$ SD) of rats (n= 10/group/day) after being exposed to surgery or sham surgery. Indices in the inner zone are measured as an index of anxiety-like behaviour observed in rats.

3.3 Experiment 3: Blood and brain tissue analysis

Following surgery, sham surgery and no intervention at all (control group), rats' trunk blood and brain tissues were collected to measure concentrations of CORT, monoamines, GABA and glutamate.

3.3.1 Blood Corticosterone analysis

Figure 3.3.1 shows a box and whisker-plot of CORT concentrations measured in the trunk blood of rats within each group (surgery or sham surgery), using radiomunoassay. The Kruskal-Wallis test detected no significant difference across time in the concentration of CORT for rats that underwent sham surgery ($p = 0.61$) and rats that underwent surgery ($p = 0.98$). There was also no significant difference in the average concentration of CORT across all four days between rats that underwent surgery and sham surgery compared to rats that had no intervention at all (Kruskal-Wallis test, $p = 0.74$, Fig 3.3.1). Therefore abdominal surgery did not induce CORT secretions in these animals.

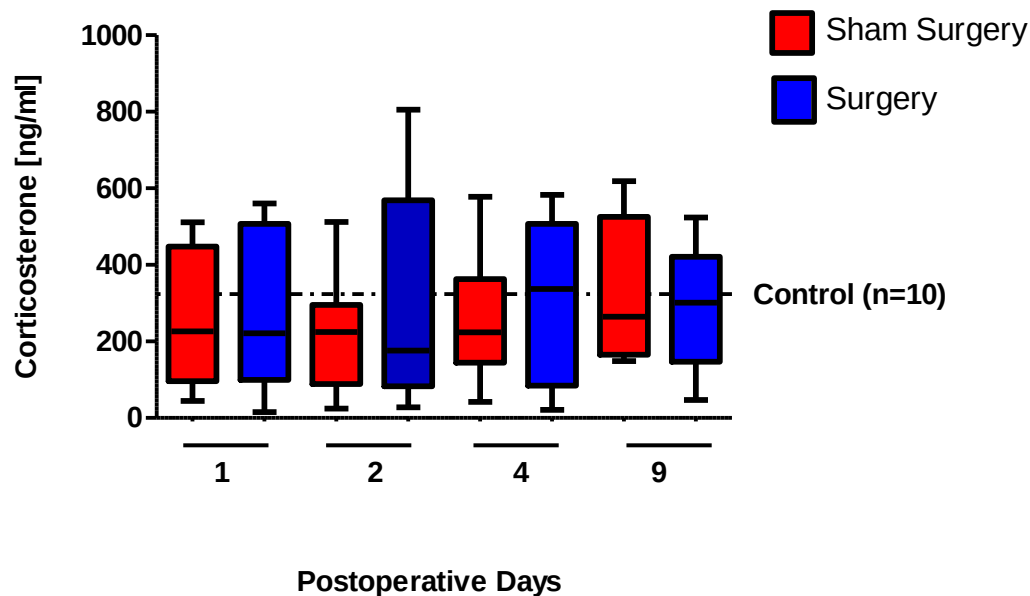


Fig 3.3.1 CORT concentrations (mean $\pm$ SD) measured in the trunk blood of rats (n=10 per group per day) on day one, two, four and nine following surgery. The dotted line represents the average concentration of CORT for rats in the control group (no intervention).

3.3.2 Prefrontal cortex monoamines

Fig 3.3.2 shows a box and whisker-plot of 5HT, NA and DA concentrations measured in the prefrontal cortex of rats, using high performance liquid chromatography. A Kruskal-Wallis test detected no significant difference in the concentration of 5HT on any of the postoperative days in the rats that underwent either sham surgery ($p = 0.94$) or surgery ($p = 0.91$). There was also no significant difference in the concentration of 5HT across all four days between rats that underwent either surgery, sham surgery or no intervention at all ($p = 0.67$). A Kruskal-Wallis test detected no significant difference in the concentration of NA across all four days between rats that underwent either sham surgery ($p = 0.7189$) or surgery ($p = 0.9107$). There was also no significant difference in the concentration of NA

across all four days between rats that underwent either surgery, sham surgery or no intervention at all ($p = 0.7575$). A Kruskal-Wallis test detected no significant difference in the concentration of DA across all four days between rats that underwent either sham surgery ($p = 0.6023$) or surgery ($p = 0.8514$). There was also no significant difference in the concentration of DA across all four days between rats that underwent either surgery, sham surgery or no intervention at all ($p = 0.6011$). Therefore, abdominal surgery did not induce significant changes in the concentration of monoamines in the prefrontal cortex.

3.3.3 Prefrontal cortex GABA and glutamate

Fig 3.3.3 shows a box and whisker-plot of GABA and glutamate concentrations measured in the prefrontal cortex of rats, using high performance liquid chromatography. A Kruskal-Wallis test detected no significant difference in the concentration of GABA across all four days between rats that underwent sham surgery ($p = 0.63$) or surgery ($p = 0.25$). There was also no significant difference in the concentration of GABA across all the four days between rats that underwent surgery, sham surgery or no intervention at all ($p = 0.90$). A Kruskal-Wallis test detected no significant difference in the concentration of glutamate across all four days between rats that underwent sham surgery ($p = 0.22$) or surgery ($p = 0.58$). There was also no significant difference in the concentration of glutamate across all four days between rats that underwent surgery, sham surgery or no intervention at all ($p = 0.96$). Therefore, abdominal surgery did not induce significant changes in the concentration of GABA and glutamate in the prefrontal cortex.

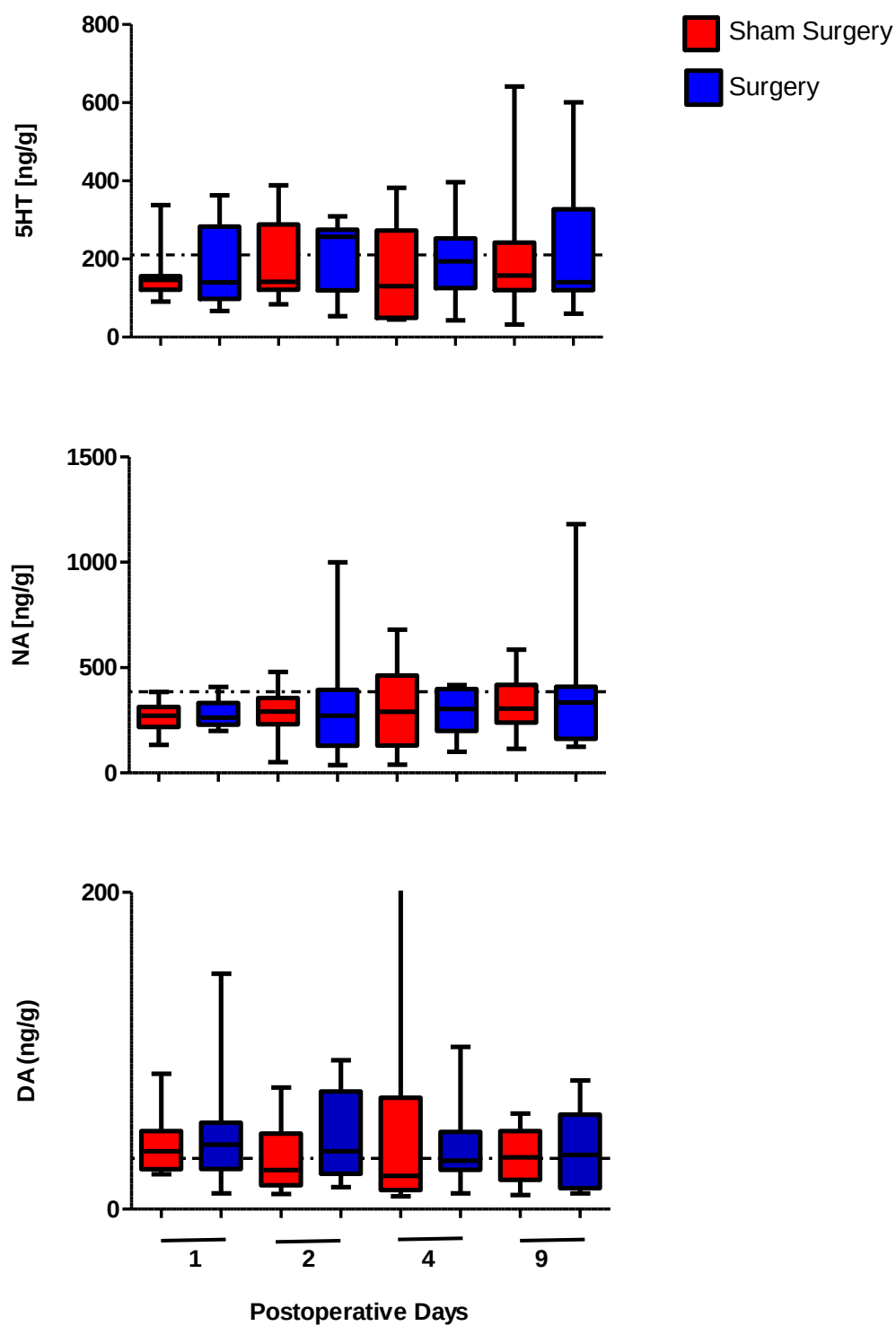


Fig 3.3.2 5HT, NA and DA concentrations (mean $\pm$ SD) within the prefrontal cortex of rats (n= 10 per group per day), measured on postoperative days one, two, four and nine, after either surgery, sham surgery or no intervention. The dotted lines represent the control group (no intervention).

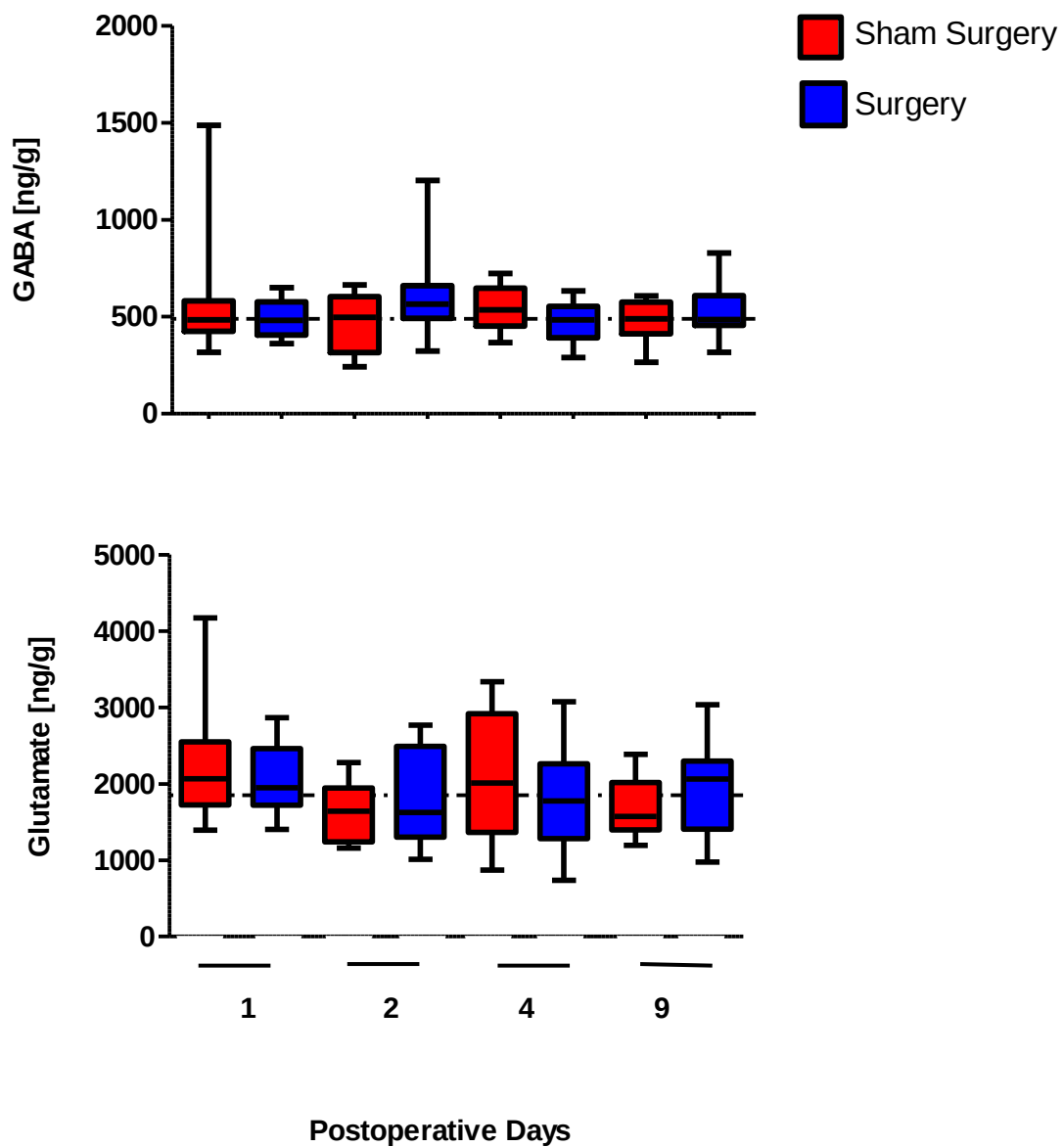


Fig 3.3.3 GABA and glutamate concentrations (mean $\pm$ SD) within the prefrontal cortex of rats ($n= 10$ per group per day), measured on postoperative days one, two, four and nine, after either surgery, sham surgery or no intervention. The dotted lines represent the control group (no intervention).

3.3.4 Hippocampal monoamines

Fig 3.3.4a shows a box and whisker-plot of 5HT and NA concentrations measured in the hippocampi of rats, using high performance liquid chromatography. A Kruskal-Wallis test detected no significant difference in the concentration 5HT across all the four days between rats that underwent either sham surgery ($p = 0.80$) or surgery ($p = 0.68$). There was also no significant difference in the concentration of 5HT across all the four days between rats that underwent either sham surgery, surgery or no intervention at all ($p = 0.66$). A Kruskal-Wallis test detected no significant difference in the concentration NA across all the four days between rats that underwent either sham surgery ($p = 0.71$) or surgery ($p = 0.50$). There was also no significant difference in the concentration of NA across all the four days between rats that underwent either sham surgery, surgery or no intervention at all ($p = 0.015$).

Figure 3.3.4b shows a box and whisker-plot of DA concentrations measured in the hippocampi of rats, using high performance liquid chromatography. A Kruskal-Wallis test showed no significant difference in the concentration of DA across all four days between rats that underwent sham surgery ($p = 0.85$) and surgery ($p = 0.23$). There was however, a significant difference in the concentration of DA in rats that underwent sham surgery vs rats that had undergone surgery and in rats that had undergone sham surgery vs rats that had no intervention at all. ($p < 0.01$). Therefore, abdominal surgery did not induce significant changes in the concentration of 5HT and NA in the hippocampi of rats. However, on the other hand, the abdominal surgery was sufficient enough to decrease the concentration of DA in surgery rats compared to sham surgery rats. It is interesting to find

that rats which had undergone no intervention at all also had a decrease in the concentration of DA compared to sham surgery rats.

3.3.5 Hippocampal GABA and glutamate

Fig 3.3.5 shows a box whisker-plot of GABA and glutamate levels measured in the hippocampi of rats, using high performance liquid chromatography. A Kruskal-Wallis test showed no significant difference in the concentration of GABA across all four days between rats that underwent sham surgery ($p = 0.95$) and surgery ($p = 0.24$). There was also no significant difference in the concentration of GABA across all four days between rats that either underwent sham surgery, surgery or no intervention at all ($p = 0.60$). A Kruskal-Wallis test showed no significant difference in the concentration of glutamate across all four days between rats that underwent sham surgery ($p = 0.93$) and surgery ($p = 0.38$). There was also no significant difference in the concentration of GABA, on any of the postoperative days, in rats that either underwent sham surgery, surgery or no intervention at all ($p = 0.32$). Therefore abdominal surgery was not sufficient enough to induce changes in GABA and glutamate concentrations within the hippocampi of rats.

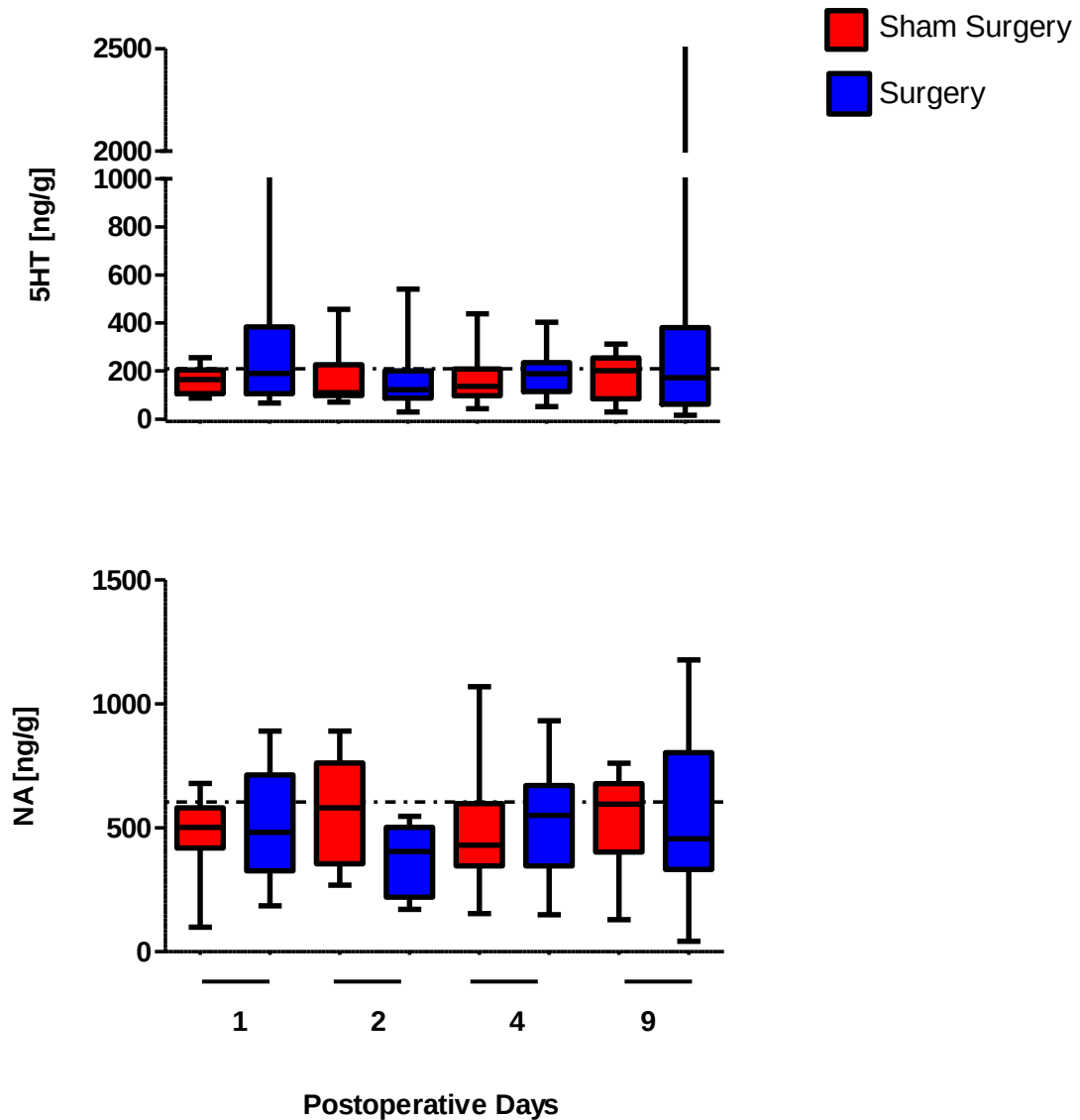


Fig 3.3.4 a 5HT and NA concentrations (mean $\pm$ SD) in the hippocampi of rats (n= 10 per group per day), measured on postoperative days one, two, four and nine, after either surgery, sham surgery or no intervention at all. The dotted line represents the control group (no intervention).

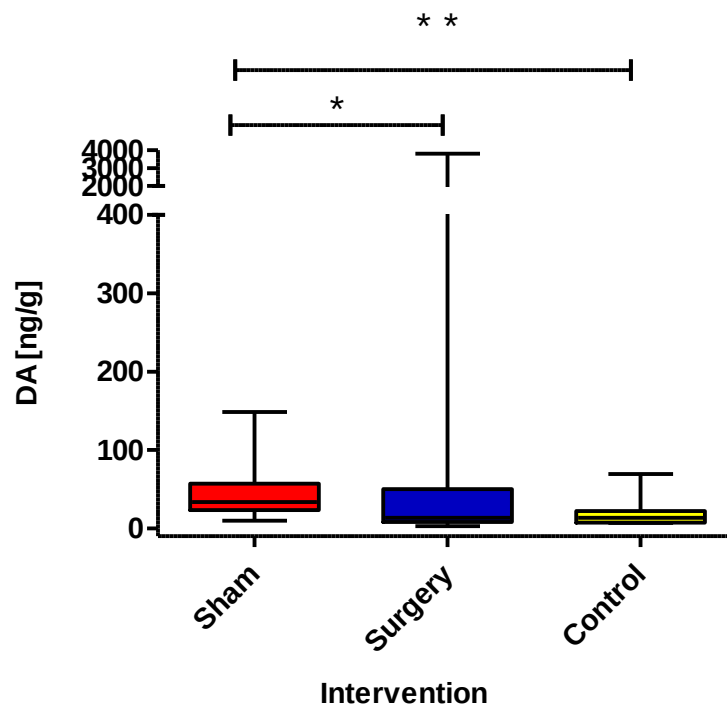


Fig 3.3.4 b DA concentrations (mean $\pm$ SD) in the hippocampi, measured in rats that underwent sham surgery (n=40), surgery (n= 40) and no intervention at all (control group) (n=10). * Sham surgery vs surgery $P < 0.01$; ** sham surgery vs control (no intervention) $P < 0.01$.

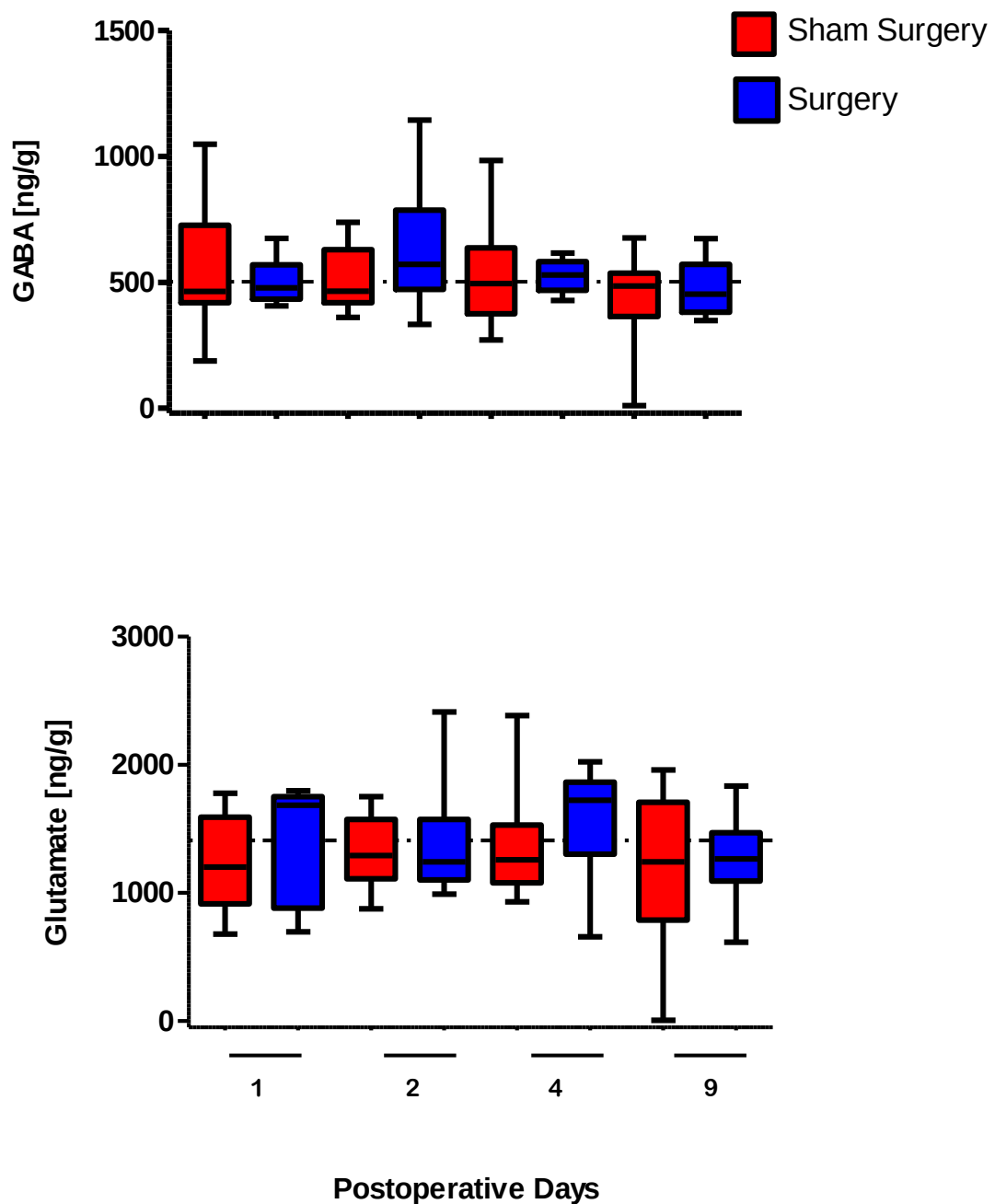


Fig 3.3.5 GABA and glutamate concentrations (mean $\pm$ SD) in the hippocampi of rats (n= 10 per group per day), measured on postoperative days one, two, four and nine, after either surgery, sham surgery or no intervention at all. The dotted line represents the control group (no intervention).

Chapter 4

Discussion

I investigated whether rats develop hypernociception and anxiety-like behaviour following abdominal surgery. I demonstrated, using standard nociceptive testing techniques, that rats display hypernociception for six days following abdominal surgery. However, the rats did not display anxiety-like behaviour post-operatively, which we tested using the open field paradigm and biomarkers of anxiety in blood and brain tissues.

4.1 Hypernociception

In several rodent model studies of postoperative hypernociception, it has been observed that an incision can cause hypernociception as soon as 30 minutes after surgery and usually is maintained for up to three to four days (Brennan, et al., 1996; Whiteside, et al., 2004; Kawamata, et al., 2005). I therefore chose to study hypernociception for six days after surgery and retested the animals on the ninth day postoperatively to determine whether the hypernociception had resolved. I found that the rats developed hypernociception for up to six days following abdominal surgery and by the ninth day the hypernociception had resolved (see fig. 3.1 in chapter 3). My study is in agreement with the study by Pogatzki *et al* (2002) in which the animals' withdrawal thresholds remained decreased for a period of eight days following surgery and then returned to normal levels. However, the incision in the study by Pogatzki *et al* (2002) was conducted in the plantar aspect of the rat foot while my incision was conducted on the rat's abdomen. It therefore seems plausible that abdominal surgery and plantar surgery have similar underlying mechanisms in the initiation and maintenance of postoperative hypernociception. Although, the mechanisms of postoperative pain have not been fully developed, it is probable that peripheral sensitization of primary nociceptive afferents and central sensitization of dorsal horn

neurons within the spinal cord are involved in the development and maintenance of the mechanical hypernociception in my study (Zhan, et al., 2003). However, I did not test these factors as they did not form part of my study. It is also important to note that the mechanical hypernociception associated with incisions in rodents, mimics the duration of mechanical hyperalgesia observed in human patients and which is exacerbated from coughing after surgery (Brennan, et al., 1996). Mechanical hyperalgesia was found in patients which had undergone inguinal herniorrhaphy and open cholecystectomy, when a force was applied directly to and adjacent to the sites of surgery (Tverskoy, 1990; Johansson, 1994).

4.2 Anxiety-like behaviour

Previous human studies have demonstrated that anxiety levels may intensify pain severity in the clinical setting (Ploghaus, et al., 2001). Moreover, studies focussing on another form of pain, neuropathic pain, have investigated the co-morbidity between pain and anxiety in rodents (Wallace, et al., 2008). Neuropathic pain differs from postoperative pain in its basic physiological mechanisms. Neuropathic pain is characterised as pain resulting from nerve trauma or damage (Wallace, et al., 2008), whereas postoperative or incisional pain is characterised from an inflammatory response which sensitizes peripheral neurons (Brennan, et al., 1996). These studies demonstrated that rats suffering from forms of neuropathic pain, had decreased hind paw withdrawal thresholds indicating hypernociception and also displayed anxiety-like behaviour within the open field paradigm (Wallace, et al., 2007a, 2007b, 2008). I therefore decided to investigate whether anxiety-like behaviour develops in a rat incision model as it did in a rat neuropathic pain model, as

my aim is to develop a better, non-invasive postoperative pain rat model that relied on measurement of complex behaviours rather than provoked nociceptive reflexes.

I hypothesised that the hypernociception experienced from surgery would be stressful enough to induce anxiety-like behaviour in rats. Using the open field paradigm I found that, up to nine days after abdominal surgery rats do not develop anxiety-like behaviour. This assessment was based on the number of inner zone entries, time spent within the inner zone and distance spent within the inner zone, which did not differ between rats that underwent surgery and those that did not. However, in a similar study conducted by Li *et al* (2010), anxiety-like behaviour was detected in a rat incision model. There are however, certain discrepancies between our two studies which may explain the difference in results. Firstly, Li *et al* used male Wistar rats whereas I used the Sprague Dawley strain. Previous studies have demonstrated differences in anxiety profiles in rats that originate from different strains (Vys, *et al.*, 2003). Secondly, Li *et al*, (2010) conducted a hind-paw incision whereas my rats underwent an abdominal incision. This is the most likely factor to have influenced the differences in our results. Rats move around by placing pressure on their paws and not their abdomens. However, like my results, Li *et al* (2010) also found that although the incision rats spent less time in the open arms of the elevated plus maze (other paradigm they used), this was not due to the incision obstructing their locomotion as the total distance they travelled was not significantly reduced. However, it is plausible that there is additional stress on an animal when it is experiencing pain in a weight-bearing body part required for locomotion.

4.2.1 The open field paradigm

Although there are countless paradigms with which to study anxiety-like behaviour, I chose the open field for my study because it has been used extensively in anxiety-related animal studies, including studies on nociception (Katz, et al., 1981; Belzung, 2001; Barbier, & Wang, 2009; Li, et al., 2010; Prut & Belzung, 2003; Slawecki, et al., 2003; Wallace, et al., 2007a,b). Furthermore, it is also easily constructed, with the materials readily available. The testing environment is easy to duplicate, while the lighting conditions are easily manipulated. The open field is a successful paradigm for the study of anxiolytics and therefore seems to be a popular choice (Belzung, 2001).

Generally when entering the open field (see fig. 2, chapter 2), rats tend to be stressed and anxious, as the arena is novel and a great deal larger than their natural environments (Prut & Belzung, 2003). However, laboratory rats did not ever experience their natural environment. They should also be anxious due to the fact that they are naturally social animals and tend to spend all their time with members of their species (Prut & Belzung, 2003) and in anxiety studies are placed individually into the arena. It must be noted however that, in my study the rats spent a great deal of time in individual cages prior to anxiety-like behaviour testing. After surgery, the rats were placed into individual cages so that they did not interfere with each others' stitches. Therefore it is possible that they were used to being alone and did not display anxiety-like behaviour within the open field or, all rats were chronically stressed from being housed individually. Li *et al* (2010), did not state how their rats were housed. Although we hypothesised that the incision itself would increase the rats' anxiety-like behaviour, this was not the case and so the

measurement of the hypernociception experienced from the incision or the site of the incision was perhaps not sufficient enough to cause sufficient distress and the development of anxiety-like behaviour in these rats.

All behavioural paradigms need to withstand three main criteria: predictive, construct and face validity, in order to be successfully related to human behaviour (Belzung, 2001). Scientists have suggested that the open field is successful in claiming face validity because when animals are placed in this paradigm they avoid a threat and humans also tend to avoid threatening situations (Belzung & Griebel, 2001; Cryan, & Holmes, 2005). The open field is also successful in claiming construct validity because stress induces anxiety-like behaviour in rats, while it also induces anxiety in humans (Prut & Belzung, 2003). However, this paradigm cannot claim predictive validity because it is not sensitive to anxiolytics that are used to treat all different types of anxiety disorders such as post traumatic stress disorder, panic disorder and obsessive compulsive disorder. Although, it is believed, that the open field paradigm does claim predictive validity for general anxiety (Prut, 2003). In other words, the open field paradigm seems to be a successful model in which to mimic anxiety that people experience when they are placed in a stressful situation as opposed to a model for people suffering from pathological anxiety (Prut & Belzung, 2003). Perhaps, another paradigm such as the elevated plus maze would have been a better choice, as it can claim predictive validity (Slawecki, et al., 2003). However, on the other hand, no one paradigm is completely perfect (Cryan & Holmes, 2005). Various studies also use a combination of paradigms. This may have been a limitation in my study. To strengthen my study, I could have used both the open field and the elevated plus maze to test anxiety-like behaviour.

Studies have also alluded to the fact that data can be misinterpreted when the open field is concerned, for example rats will tend to explore the arena more if they are food and water deprived, therefore increasing their exploration for that reason and not because they are anxious (Pruet & Belzung, 2003). In my study rats were neither food nor water deprived therefore that factor did not pose a problem. Moreover, lighting conditions are very important when using the open field to study anxiety-like behaviour. Studies have shown that when rats are placed in bright lighting conditions they display an increase in anxiety-like behaviour (Lang, et al., 2000) because they are nocturnal animals. This was unfortunately not a factor I took into account when conducting my study, although the open field paradigm was situated in a room where the lights were switched on, and the intensity of the light was 200lux. Perhaps, the lighting conditions were not bright enough to induce anxiety-like behaviour. Although natural lighting conditions, which rats are exposed to on a bright day, are much brighter than the standard office lighting I used in my study, we still need to consider the fact that rats are active at night and not during the day.

Along with anxiety-like behaviour, I tested the rats' rearing behaviour. Rearing is a behaviour displayed by rats when curiously exploring a novel environment for means of escape (Landgraf & Wigger, 2002). It is defined as a vertical movement when animals need to rear on their hindlegs (Martin, et al., 2004). Studies have shown that rats which display an increased anxiety-like behaviour within the open field tend to demonstrate decreased rearing behaviour (Katz, et al., 1981). I firstly hypothesised that rats which had undergone surgery would rear less than the rats that had not undergone surgery because these rats would be experiencing hypernociception which would increase their anxiety-like behaviour within the open field. Landgraf & Wigger (2002), on the other hand, demonstrated that rats which display high anxiety behaviour display less rearings than rats

which display low anxiety behaviour. Secondly, I believed that rats that had undergone surgery would be experiencing increased levels of hypernociception and this would decrease their ability to expand their abdominal musculature when rearing. In a study by Martin *et al* (2004), rats that had undergone a laparotomy reared less on postoperative day one, two and four compared to sham surgery that did not undergo surgery. By postoperative day seven, these laparotomized rats reared the same amount of times as sham surgery rats. Therefore, indicating that the effects of hypernociception had resolved by postoperative day seven. In my study, the incised rats seemed to rear the same amount as those that were not incised on all the postoperative days investigated. Therefore, the hypernociception that these rats experienced was not sufficient enough to produce increased anxiety-like behaviour and a decrease in the rats' rearing behaviour after surgery.

On the other hand, one might suggest that the anaesthesia which the rats received prior to surgery or the pain medication given to the rats following surgery may have influenced the lack of anxiety-like behaviour in these rats when placed in the open field. This is unlikely because firstly, the rats were not given any pain medication following surgery for this very reason. Secondly, if the anaesthesia had influenced the rats' behaviour, one would expect their anxiety-like behaviour to be decreased one day following surgery and by the second day onwards, when the anaesthesia had resolved, the anxiety-like behaviour would have increased. However, there was no significant difference in anxiety-like behaviour profiles between incised rats and non-incised rats on any of the postoperative days.

4.3 Anxiety biomarkers

I did not manage to detect anxiety-like behaviour, using the open field paradigm, in my study. I therefore wanted to determine whether the lack of anxiety-like behaviour was due to my actual model which was just not sensitive enough to detect the anxiety or whether rats just do not experience anxiety-like behaviour after abdominal surgery. My next step was therefore to investigate different anxiety biomarkers in the rat brain and blood which would provide me with an actual anxiety profile or lack thereof. I chose to investigate the three monoamines, serotonin, dopamine, noradrenaline; and also GABA and glutamate in my study, because previous studies have implicated all these neurotransmitters in the development and maintenance of anxiety and anxiety-like behaviour in humans and rats respectively (Gordon & Hen, 2004; Mallo, et al., 2007).

Secondly, I chose to study the levels of these neurotransmitters within the prefrontal cortex and the hippocampus. Many anxiety studies have been used to investigate the levels of neurotransmitters within these two areas, along with other areas associated with anxiety behaviour (Connor & Davidson, 1998; Ploghaus, et al., 2001; Kalinichev, et al., 2002). Both areas have been implicated in producing an increased anxiety state when a stressor is applied (Hsu, et al., 2007). The hippocampus and prefrontal cortex are also relatively easy to dissect and well suited for the measurement of neurotransmitter levels associated with anxiety.

4.3.1 Monoamines, GABA and glutamate in the Hippocampus and Prefrontal Cortex

The hippocampus is responsible for pain processing, memory retrieval and encoding (Melzack & Casey, 1968). Studies have implicated the hippocampus in anxiety-induced hyperalgesia (Ploghaus, et al., 2001). According to the Gray-McNaughton theory, there is a strong correlation between anxiety and increased pain processing within the hippocampal formation (Gray & McNaughton, 2000). Parts of the prefrontal cortex have also been implicated in inducing anxiety and fear behaviour during stressful situations (Vys, et al., 2003; Hsu, et al., 2007). The prefrontal cortex is believed to be one of the sites for the interpretation of experiences associated with anxiety (Connor & Davidson, 1998; Kalinichev, et al., 2002).

I expected to find either an increase or decrease in 5HT levels within the hippocampus and prefrontal cortex, if the rats were to be anxious. This is a strange hypothesis stemming from the controversy between studies regarding 5HT and anxiety. Kagamiishi *et al* (2003) suggested that 5HT_{1A}R receptors are found mainly within the hippocampus and are involved in the induction of anxiety, while 5HT_{2R} receptors populate mainly the prefrontal cortex. 5HT_{1A} receptor agonists decrease the release of 5HT in target tissues and therefore provide as anxiolytics tested within the open field and elevated plus maze (Gordon & Hen, 2004). A study by Matsuo *et al*, (1996) demonstrated that an increase in 5HT within the hippocampus, due to a conflict situation in the Vogel type conflict test, was associated with anxiety-like behaviour (Matsuo, et al., 1996). However, when a 5HT_{1A} agonist was injected into the hippocampus, anxiogenic effects were seen (Overstreet, et al., 2003). It has also been shown that 5HT which is released into the hippocampus also

decreases anxiety-like behaviour (Ferguson, et al., 2004; Gordon & Hen, 2005). Another investigation concentrating on gene knockouts, demonstrated that mice with a MAOA gene knockout had increased levels of 5HT in both the hippocampus and prefrontal cortex and an increase in fear behaviour (Holmes, 2008). In my study, I found no significant difference in 5HT concentrations within the hippocampus and prefrontal cortex between the surgery, sham surgery or control animals on any of the postoperative days. Therefore, suggesting that the rats did not experience anxiety-like behaviour following surgery. Neither the surgery itself nor the hypernociception associated with the incision were sufficient enough to produce anxiety-like behaviour in these rats.

I also expected to find an increase in DA and NA within the hippocampus and prefrontal cortex, if our rats were to be anxious and stressed following surgery. Studies have demonstrated that acute stressors such as tail-pinch stress increase NA concentrations within the hippocampus and prefrontal cortex, along with increased anxiety-like behaviour within the open field paradigm (Harvey, et al., 2006; Mallo, et al., 2007). Other acute stressors such as the water immersion test and restraint stress and others cause an increase in DA levels within the rat hippocampus and prefrontal cortex (Rossetti, et al., 1990; Pani, et al., 2000; Harvey, et al., 2006). It has even been suggested that mild stress increases DA release within the prefrontal cortex to a greater extent than other associated regions (Dazzi, et al., 2001). In my study however, I found no significant difference in NA concentrations within the hippocampus and prefrontal cortex between surgery, sham surgery and control animals on any of the postoperative days. Suggesting that, surgery was not sufficient enough to produce stress and anxiety-like behaviour in the rats. However, there was a significant difference in DA concentrations between the sham surgery group versus the

surgery group and the sham surgery group versus the control group. I believe this result to be an artifact.

I also found no significant difference in hippocampal GABA and glutamate concentrations between surgery, sham surgery and control animals. Previous studies have demonstrated increased GABA release in the hippocampus following forced swimming, which is an established acute stressor test (Harvey, et al., 2004). One of the functions of GABA within the brain is the attenuation of excess glutamatergic activity (Harvey, et al., 2004). Harvey *et al*, (2004) have even suggested that patients that have irregularities with their GABAergic signal transduction within the hippocampus or prefrontal cortex may be predisposed to anxiety disorders (Harvey, et al., 2004). Male rats that have been stressed by maternal separation also tend to demonstrate a decrease in GABA_A receptors in the prefrontal cortex (Kalinichev, et al., 2002). I can therefore speculate that the hypernociception stemming from neither the incision nor the surgery itself are stressful enough to induce anxiety-like behaviour within the open field nor elevate the GABA and glutamate levels within the hippocampus.

4.3.2 Corticosterone concentration in the blood

I decided to study blood CORT concentrations in the rat because of the strong correlation between stress and anxiety in other studies. Rats that are stressed by certain stimuli tend to produce more CORT, the stress hormone, along with experiencing increased anxiety-like behaviour (Shepard, et al., 2000; Barbier & Wang, 2009; Kalinichev, et al., 2002). Studies have shown that CORT implants in the amygdala are related to a decrease in open arm

preferences in the EPM, which suggests an anxiety-like behaviour profile (Shepard, et al., 2000). I hypothesised that rats after abdominal surgery would have an increase in blood CORT levels because the hypernociception they experienced would cause them stress. The surgery itself could have also been stressful and so we expected an increase in their CORT levels. This was however not the case. Rats that underwent an incision showed no significant difference in their blood CORT levels compared to sham surgery rats or even control rats which had no surgery at all. Therefore suggesting that abdominal surgery or the hypernociception associated with this surgery is not a stressful event for rats. However, it is unlikely that surgery itself is not stressful for rats and so it is plausible to suggest that the timing of my sampling may have affected my results. Perhaps if the brain and blood samples were collected at an earlier time then my results may have been different.

Chapter 5

Conclusion

Much research is focused on the pharmacological aspects of treating postoperative pain, but without basic research using animal models, much of the necessary pain mechanisms would not be discovered and in turn the pharmacological targets would be unknown. My aim was to develop a non-invasive rat model of postoperative pain by using anxiety models. However this was not possible as the rats did not display anxiety-like behaviour.

In conclusion, although male Sprague-Dawley rats display hypernociception for six days follow abdominal surgery, tested using standard nociceptive techniques, they do not display anxiety-like behaviour following surgery, tested using the open field paradigm. Moreover, the latter result was further iterated by anxiety biomarker studies, which showed that rats after surgery do not display a difference in neurotransmitter and stress hormone concentrations compared to presurgical concentrations. My study showed not only that rats that undergo abdominal surgery do not necessarily display anxiety-like behaviour, but also that the open field might not be a sensitive enough paradigm to detect anxiety-like behaviour following surgery. Also, different areas of incision may, or may not, induce different degrees of anxiety. Future research needs to focus on different models and paradigms of anxiety-like behaviour by perhaps developing a study which compares rats after abdominal surgery in different anxiety paradigms, such as the open field, the light/dark box test and elevated plus maze, as these paradigms may be more sensitive in establishing anxiety-like behaviour after surgery. Presurgical anxiety has been correlated with increased severe postoperative pain, which in turn, is a great concern as it causes prolonged hospital stays and readmissions that are expensive. We need to continue conducting postoperative pain and anxiety studies in order to investigate the basic pain and anxiety mechanisms and therefore develop appropriate analgesics and anxiolytics.

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APPENDIX 1

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2010/20/04

APPLICANT: Ms S Ferreira

SCHOOL: Physiology

DEPARTMENT:

LOCATION:

PROJECT TITLE: Is postoperative pain with anxiety like behaviour in rats

Number and Species

3 (pilot study), 110 rats

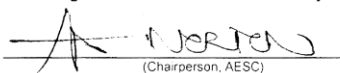
Approval was given for the use of animals for the project described above at an AESC meeting held on 30.03.2010. This approval remains valid until 30.03.2012.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Please

- confirm the site of the surgical incision with the CAS veterinary surgeon,
- consider providing overnight analgesia. This should be discussed and agreed on with the CAS veterinary surgeon

Signed: _____


(Chairperson, AESC)

Date: _____

10/04/2010

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____


(Registered Veterinarian)

Date: _____

16/04/2010

cc: Supervisor:
Director: CAS

Works 2000/1ain0015/AESCCert.wps



STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2011/12/04

APPLICANT: Ms S Ferreira

SCHOOL: Physiology

DEPARTMENT:

LOCATION: Medical School

PROJECT TITLE: Are biomarkers of stress and anxiety increased in rats after surgery?

Number and Species

115 rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 20110222. This approval remains valid until 20130221.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

1. Five rats for pilot project; 10 rats per experimental group; 10 rats for control group
2. Must be a control group (of 10 rats)
3. Rate of approximately 200 grams body weight to be used
4. Three minutes maximum for entire procedure from anaesthetic to decapitation
5. HPLC, not PCR, to be used

Signed: _____

(Chairperson, AESC)

Date: _____

04/03/2011

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____

(Registered Veterinarian)

Date: _____

07/03/2011

cc: Supervisor: Professor P Kamerman
Director: CAS

Works 20001a00015/AESCcert.wps