

**A CHARACTERIZATION AND VALIDATION OF THE
PYROGENIC MODELS AND THERMOMETRIC
METHODS USED FOR PHARMACOGNOSTIC
EVALUATION OF ANTIPYRETIC MEDICINE**

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of
Doctor of Philosophy

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DECLARATION

I, Rachael Dangarembizi, declare that this thesis was solely composed by myself, that the work contained herein is my own and that any assistance has been acknowledged and all sources duly referenced. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

.....
Rachael Dangarembizi

Signed on this.....day of.....2018

DEDICATION

To my late mother.

PUBLICATIONS AND PRESENTATIONS

Publications arising out of this work

1. **Dangarembizi, R.**, Erlwanger, K H., Rummel, C., Roth, J., Madziva, M T and Harden, L M. 2018. Brewer's yeast: A potent inducer of fever and sickness behaviour through activation of inflammatory mediators in the brain. *Brain Behaviour and Immunity* **68**, 211-223.
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Conference presentations

1. **Dangarembizi, R.** Erlwanger, K H., Rummel, C., Roth, J., Madziva, M T and Harden, L M. Zymosan, a cell wall component of *Saccharomyces cerevisiae* induces fever and sickness responses through inflammatory mediators in the brain. A poster presentation at the 38th Congress of the International Union of Physiological Sciences, 1-5 August 2017, Rio de Janeiro, Brazil
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ABSTRACT

The development of safe and effective antipyretic and anti-inflammatory drugs remains an important focus of the pharmacological industry. Despite reports that rectal thermometry causes stress-induced hyperthermia in rodents, it remains the most commonly used method for measuring core body temperature during antipyretic drug-testing. The impact of the stress-induced hyperthermia on the accuracy and reliability of thermal responses reported in pharmacological studies investigating the efficacy of antipyretic drugs remains unclear. Additionally, the sickness response to Brewer's yeast, the pyrogen recommended for inducing fever during antipyretic testing, remains largely uncharacterised and its mechanisms unknown.

I used intra-abdominally implanted temperature-sensitive radiotelemeters to measure changes in the abdominal temperature of both normothermic and febrile rats injected with Brewer's yeast, and exposed to rectal temperature measurement at hourly intervals for five hours. My results show that rectal temperature measurement is associated with a reproducible, non-decremental rise in abdominal temperature (0.6 - 0.8 °C) in normothermic rats. The hyperthermia was muted in febrile rats and was not blunted by the common habituation procedure. A comparison of temperatures measured using rectal thermometry with those measured using biotelemetry showed that rectal temperatures recorded using a thermocouple probe could be up to 0.5 °C lower or 0.7 °C greater than abdominal temperatures recorded using an abdominally implanted telemeter. The differences in thermal responses exhibited by normothermic vs febrile rats, coupled with the under- or overestimation of core body temperatures when one uses thermocouple probes supports my hypothesis that the procedure of rectal thermometry may affect the accuracy of thermal responses observed during antipyretic drug-testing.

Having examined the methods used for temperature measurement, my second objective was to characterise the sickness response to the recommended and commonly used pyrogen for antipyretic screening; *Saccharomyces cerevisiae* (Brewer's yeast). I measured the change in abdominal temperature, nocturnal activity, food intake and body mass gain in male Sprague Dawley rats injected subcutaneously with one of three doses of Brewer's yeast (0.4, 0.2 and 4 g/kg). I further characterised the spatiotemporal activation of inflammatory mediators in the periphery (tumor necrosis factor alpha and interleukin (IL)-6) and in the brain (nuclear factor

(NF) for interleukin 6, NF- κ B, cyclooxygenase-2 (COX-2) and the signal transducer and activator of transcription (STAT)-3 in the vascular organ of the lamina terminalis (OVLT). Lastly, I measured the expression of hypothalamic inflammatory genes including: cytokines (IL-1 β , IL-6, TNF- α), enzymes (COX-2 and microsomal prostaglandin synthase (mPGES)) and regulators of transcription factors (NF- κ B inhibitor alpha (I κ B α) and suppressor of cytokine signalling 3 (SOCS3)). My results show that Brewer's yeast dose-dependently induces fever, lethargy, anorexia and body mass stunting. However, the sickness response to the high dose of Brewer's yeast; the dose commonly used during antipyretic screening, was associated with an initial short-lived hypothermia, lengthy fevers, excessive peripheral and central inflammation and the development of an abscess.

My last objective was to investigate if zymosan, a cell wall moiety of Brewer's yeast could be used as an alternative pyrogen during antipyretic drug testing. Using the same methods as outlined for my second study, I therefore characterised the sickness responses and activation of inflammatory mediators associated with subcutaneous injection of two doses of zymosan (300 mg/kg and 30 mg/kg). My results show that zymosan dose-dependently induces fever, lethargy, anorexia and body mass stunting and that zymosan activates the same suite of inflammatory mediators as Brewer's yeast albeit on a more moderate scale. Thus, zymosan could be used as a pyrogen for simulating fungal infections during antipyretic drug testing.

In conclusion, the studies undertaken as part of my PhD have shown that among the methods and models currently recommended for use during antipyretic drug testing, rectal thermometry is associated with stress and hyperthermia which can potentially affect the accuracy and reliability of observations and conclusions drawn on the efficacy of test drugs. Additionally, at the recommended doses, Brewer's yeast induces fever and sickness behaviours albeit with undesirable animal welfare challenges such as hypothermia, excessive inflammation and a lengthy sickness response due to the development of an abscess. However, zymosan is suitable for use as alternative to Brewer's yeast as it is capable of inducing fever, sickness behaviours and inflammation of a moderate magnitude, which would allow pharmacological interventions without inducing animal suffering related to an excessive and prolonged inflammation.

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“He raiseth up the poor out of the dust, and lifteth up the beggar from the dunghill, to set them among princes, and to make them inherit the throne of glory: for the pillars of the earth are the LORD'S, and He hath set the world upon them.” 1 Samuel 2:8

Thank you God, I owe all of my successes to your loving kindness.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AD	anno domini
BAT	brown adipose tissue
BBB	blood brain barrier
BC	Before Christ
CLR	C-type Lectin Receptor
COX	Cyclooxygenase
cPLA ₂	cytoplasmic Phospholipase A ₂
CR3	complement receptor 3
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
CVO	circumventricular organ
DMH	dorsomedial hypothalamus
DNA	deoxyribonucleic acid
DP1	prostaglandin D 1 receptor
EP	prostaglandin E receptor
GABA	gamma amino butyric acid
HPA	hypothalamic pituitary axis
IFN	interferon
I κ B α	NF κ B inhibitor alpha
IL	interleukin
IL-1R	interleukin -1 receptor
IL-1ra	interleukin -1 receptor antagonist
IRAK	interleukin-1 receptor associated kinase
JAK	Janus Kinase
KO	knock out
LC	locus coeruleus
LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinases
mIL6R	membrane-anchored interleukin 6 receptor
MnPO	median preoptic nucleus
mPGES	microsomal prostaglandin E synthase
mPOA	medial preoptic area
mRNA	messenger RNA
MyD88	Myeloid Differentiation Primary Response Protein 88
NF-IL6	nuclear factor for interleukin 6
NF κ B	Nuclear factor Kappa-light-chain-enhancer of activated B cells
NSAIDs	non-steroidal anti-inflammatory drugs
OVLТ	organum vasculosum laminae terminalis or vascular organ of the lamina terminalis

PAG	periaqueductal grey
PAMP	pathogen associated molecular pattern
PG	prostaglandin
PI3	phosphatidylinositol 3
PLA ₂	phospholipase A ₂
Poly I:C	polyinosinic: polycytidilic acid
PRR	pattern recognition receptors
PVN	paraventricular nucleus
RNA	ribonucleic acid
rRPa	rostral raphe pallidus
RT-PCR	Real Time - Polymerase Chain Reaction
sIL6R	soluble interleukin 6 receptor
sPLA ₂	secretory phospholipase A ₂
STAT	signal transducers and activators of transcription
sTNFR	soluble tumor necrosis factor receptor
TACE	tumor necrosis factor- α converting enzyme
TLR	toll like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	tumor necrosis factor-receptor associated factor
TXA ₂	thromboxane A ₂

PREFACE

Humans are constantly and unavoidably exposed to various disease-causing microorganisms, which include bacteria, viruses, fungi and protozoa. In order to improve our chances of survival, we have an in-built immune system, which fights these microorganisms. Natural and non-specific barriers including the skin, mucous membranes and various secretions such as earwax, tears and stomach acid strategically fight the entry of these potentially harmful microorganisms at all possible points of entry. In the event that these barriers are breached, the microorganisms encounter a second line of defence comprising a group of proteins and phagocytic cells that are activated to neutralize the invading pathogen and, most importantly, to inform the body about the presence of these unwanted and potentially life-threatening invaders. Normally, a local inflammatory response is triggered, which is characterised by pain, redness, heat and swelling at the site of infection. Through specialized inflammatory mediators, the immune system also communicates with the brain, the higher command-centre for controlling body function and behaviour, to adjust bodily function in accordance with the presence of the unwanted pathogens.

Upon receiving input from the immune system, the brain initiates a set of physiological, metabolic and behavioural responses which commonly include fever and sickness behaviour (a collection of non-specific signs of sickness including tiredness, loss of motivation, anorexia and depression-like behaviour). Although fever and sickness behaviour are generally regarded as beneficial, an excessively prolonged and exaggerated sickness response may negatively impact on our quality of life and often drives us to seek relief, usually in the form of antipyretic drugs. Since antiquity, mankind has used antipyretic plants such as the willow to deal with inflammation, fever and sickness. Today, several highly effective antipyretic drugs have been developed, albeit with some exhibiting undesirable side effects. Pharmaceutical companies and scientists, therefore, continue to search for safe but effective drugs and, most importantly, for better methods and models of testing antipyretic drugs.

The main aim of this thesis was therefore to systematically characterise and validate the current methods and models that are used in testing antipyretic drugs. In **Chapter 1**, I review literature on the studies that have thus far described the methods and pyrogens used in testing the efficacy and mechanisms of action of antipyretic drugs. In **Chapter 2**, I compare the two methods commonly used for measuring temperature during antipyretic testing; rectal thermometry and

biotelemetry. **Chapter 3** investigates the sickness and inflammatory responses induced by subcutaneously injecting rats with Brewer's yeast, the pyrogen that is recommended for use during antipyretic drug testing. In **Chapter 4**, I investigate whether zymosan, a moiety of Brewer's yeast can be used as an alternative pyrogen to the highly potent Brewer's yeast. The experimental findings presented in my thesis chapters, limitations of my study and recommendations for future studies are summarized and discussed in the concluding chapter, **Chapter 5**.

CHAPTER ONE - INTRODUCTION

The innate immune system comprises of physical and chemical barriers and specialized, non-specific phagocytic cells (e.g. macrophages, natural killer cells, neutrophils and dendritic cells) which are the body's first line of defence and resistance against invading pathogens (Parham, 2014). Activation of the innate immune system is often accompanied by an inflammatory response and an increase in the circulating levels of chemical messengers e.g. cytokines, leukotrienes and prostaglandins, which are mediators of the body's defensive response to infection. Circulating inflammatory mediators activate changes in the chemical milieu of the brain which trigger the development of physiological responses e.g. fever as well as behavioural responses such as lethargy, depression, loss of appetite, pain, somnolence which are collectively known as sickness behaviour (Kelley et al., 2003, Kent et al., 1988, Dantzer, 2004). Fever and sickness behaviour collectively form the sickness response or sickness syndrome, which is a crucial part of the host immune defence against infection (Hart, 1988).

Although now known to be a strategic and necessary part of the body's defence against invading pathogens, the sickness response is often paradoxically associated with discomfort and a temporary reduction in the quality of life and productivity that most would rather do without. Common practice is to therefore prescribe antipyretic and anti-inflammatory drugs that reduce the magnitude of the inflammatory response and attenuate the fever and sickness behaviour. Most antipyretic and anti-inflammatory drugs inhibit the production of various inflammatory mediators such as prostaglandins (Vane and Botting, 2003, Vane, 1971, Anderson, 2008). Other mechanisms include inhibiting non-prostaglandin dependent processes such as neutrophil migration, inhibiting the production of other inflammatory mediators (e.g. cytokines) and enhancing the production of anti-inflammatory signals [see Aronoff and Neilson (2001) for review].

Over the past century, the demand for antipyretic drugs such as aspirin and paracetamol, as common household drugs, has significantly grown and the pharmaceutical industry continues searching for new drugs that will be cheaper and more effective. Despite recent technological advances, the methods and models used for testing antipyretic drugs have not changed much over the past almost 100 years and the mechanisms underlying the sickness response induced by the pyrogen recommended for inducing fever during antipyretic testing remain largely uncharacterised.

This introductory chapter summarizes the development of fever and sickness behaviour following infection and the use of antipyretic drugs to alleviate the maladaptive symptoms of this sickness response. I then review the current methods and models used in testing antipyretic drugs and reveal gaps in our knowledge, which will be addressed by the experiments described in this thesis.

1.1 The classical mechanisms known to mediate fever and sickness behaviours

1.1.1 Pathogen recognition

The entry of an exogenous pyrogen into the body is immediately detected by non-specific, phagocytic cells of the innate immune system. These cells express pattern recognition receptors (PRRs), e.g. toll like receptors (TLRs) and C-type lectin receptors (CLRs), which allow for the identification of structures that are conserved in microbial species, commonly known as pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) [see (Takeuchi and Akira, 2010) for review]. Different PRRs recognize different molecular patterns on microorganisms e.g. TLR2 forms heterodimers with TLR1 and TLR6 and distinctly recognizes diacyl and triacyl lipoproteins from bacterial, mycoplasma and fungal cells (Takeuchi and Akira, 2010) while TLR3 recognizes double-stranded viral RNA (Matsumoto and Seya, 2008) and TLR4 recognises the Gram-negative bacterial endotoxin lipopolysaccharide (LPS) as well as some viral envelope proteins (Lu et al., 2008, Saito and Gale, 2007). The Dectin family, a subgroup of CLRs, recognizes carbohydrate molecules, such as glucans, chitins and mannans mostly found in fungal microbial species (Saijo and Iwakura, 2011). The response of each PRR has been shown to differ depending on the type of ligand that binds, as well the type of immune cell that is activated. In general, stimulation of PRRs activates the innate immune system and increases the production of inflammatory mediators, such as chemotactic factors, interferons, chemokines, acute phase proteins and, cytokines, which play a role in the development of fever and sickness behaviour (Takeuchi and Akira, 2010, Akira et al., 2006).

1.1.2 Pro-inflammatory cytokines

Cytokines are inducible, pleiotropic polypeptide molecules released by immunocompetent cells, whose primary function is to regulate and coordinate immune responses (Conti et al., 2004, Striz et al., 2014). Although cytokines have been extensively studied, we remain far from knowing the dynamics of the full mechanisms underlying their function during an immune response to infection, partly due to the conflicting descriptions of their biological effects given in literature [for examples see (Kawasaki et al., 1989, Leon et al., 1997, Long et al., 1990b, Roth et al., 1998, Töllner et al., 2000b, Shachar and Karin, 2013)]. These conflicting findings could be attributed to differences in (1) species used (e.g. rats, mice, rabbits, guinea pigs), (2) the nature of the exogenous pyrogens administered (e.g. bacterial vs. viral or fungal, and serotypes of each microbe, purity of pyrogens etc.) and (3) the routes of administration or the models of inflammation used (e.g. acute vs. chronic, systemic vs. local). Moreover, the experimental approach for investigating the function of a cytokine is usually to block its release or its function and observe how the immune response is altered. However, it remains a challenge to fully delineate the individual contribution of each cytokine to the immune response or the hierarchical action of a group of cytokines due to cross-inductions and compensatory functions exhibited when the blocked cytokine is absent. Be that as it may, research has still managed to unravel some of the functions of individual cytokines and because some of these cytokines (interleukin-1 β , interleukin-6 and tumor necrosis factor- α) are implicated in the sickness responses discussed in **Chapters 3 and 4** of this thesis, I will briefly discuss their contribution to the development of febrile responses to infectious agents. The common inflammatory mediators and pathways activated by each of these three cytokines are summarized in Figure 1.1.

1.1.2.1 Interleukin-1 β

Interleukin-1 β (IL-1 β) is one of the most extensively studied members of the 11-member IL-1 family, and is released by immunocompetent cells such as macrophages and microglia during infection (Vitkovic et al., 2000). IL-1 β binds to the type I IL-1 Receptor (IL-1RI) and initiates the transduction of inflammatory signals through the recruitment of the myeloid differentiation primary response protein (MyD88), the IL-1 receptor

associated kinase (IRAK), the TNF- α -receptor associated factor (TRAF) and subsequent activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) (Malinowsky et al., 1995, Jefferies et al., 2001).

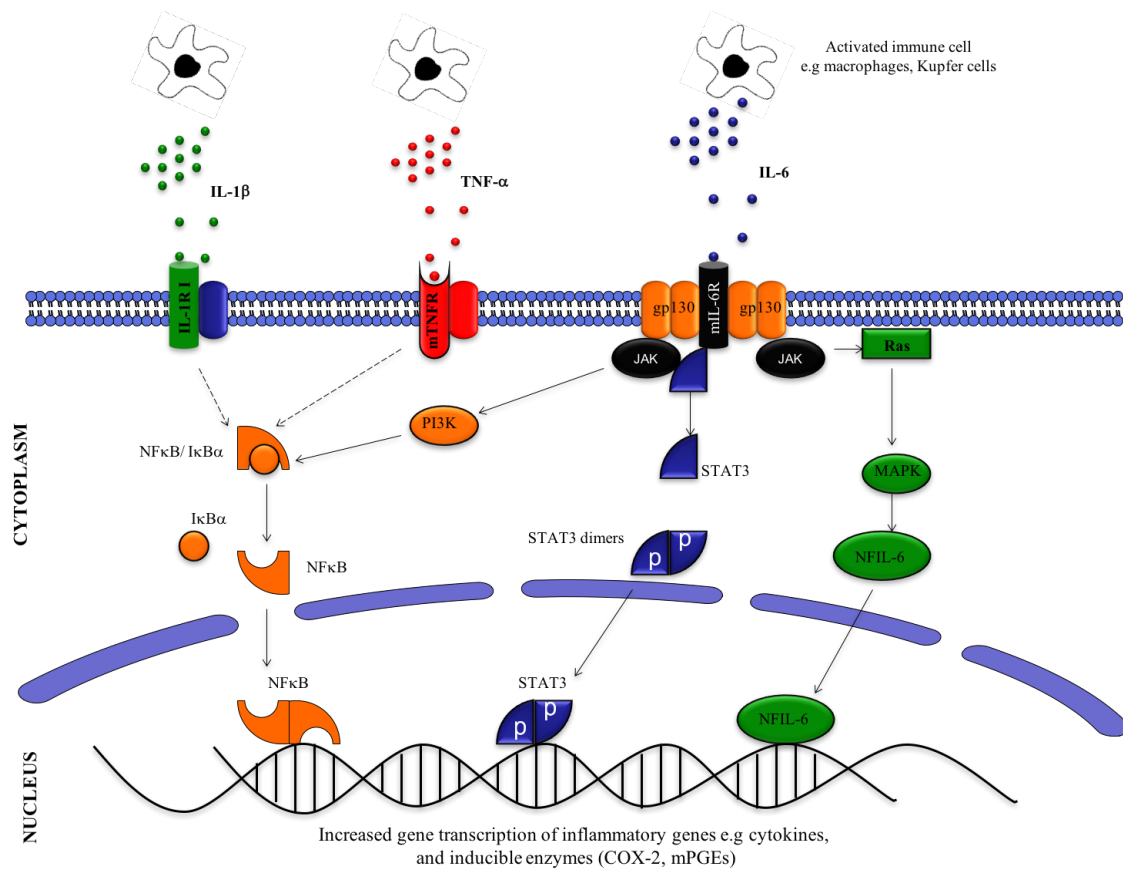


Figure 1.1: Intracellular signalling pathways activated by the binding of IL-1 β , IL-6 and TNF- α to their respective receptors on a target cell. IL-1 β binds to a membrane-anchored IL-1R (mIL-1R) receptor and TNF- α binds to a membrane-bound TNF (mTNFR) receptor. Binding of IL-1 β and TNF to their respective receptors leads to the dissociation of I κ B α from NF- κ B and the subsequent translocation of NF- κ B into the nucleus where it activates transcription of genes encoding inflammatory molecules e.g. cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase (mPGES). IL-6 binds to its membrane-bound IL-6 receptor (mIL-6R) or alternatively to a soluble IL-6 receptor (not shown) which associates with gp130 and activates Janus Kinase (JAK) and the subsequent activation, dimerization and nuclear translocation of the signal transducer and activator of transcription (STAT) which in turn activates transcription of inflammatory genes. The binding of IL-6 also activates the Ras/Mitogen activated protein kinases (MAPK) pathway leading to the activation and nuclear translocation of the nuclear factor for IL-6 (NFIL-6) and subsequent transcription of inflammatory genes. Through the activation of the JAK/phosphatidylinositol-3 Kinase (PI3K) pathway, IL-6 binding also activates NF- κ B.

(Figure 1.1 was generated by Rachael Dangarembizi from basic principles of intracellular cytokine signalling)

When activated, NF- κ B dissociates from its inhibitory regulator I κ B α and translocates to the nucleus where it promotes the transcription of a number of inflammatory genes e.g. cytokines, immunoreceptors, enzymes such as cyclooxygenase-2 as well as its inhibitory regulator I κ B α (Nadjar et al., 2005, Sun et al., 1993). Because inflammatory signals activate NF- κ B and promote its translocation into the nucleus and subsequent synthesis of I κ B α , several studies have shown that staining for nuclear NF- κ B and monitoring I κ B α synthesis can serve as useful tools of measuring neuroinflammation (Laflamme and Rivest, 1999, Quan et al., 1997). IL-1 β also binds to the type II IL-1 Receptor (IL-1RII) but this receptor is now known to be a decoy receptor, believed to negatively regulate the function of the cytokine (Mantovani et al., 2001).

The pyrogenic effects of IL-1 β have been demonstrated in studies showing that fever is attenuated or inhibited in: (1) IL-1 β knockout mice injected with turpentine (Horai et al., 1998, Zheng et al., 1995) or LPS (Kozak et al., 1998), and (2) rats injected with IL-1 receptor antagonist (IL-ra) after injection of the viral mimic, polyinosinic: polycytidylic acid (poly I:C) (Fortier et al., 2004, Luheshi et al., 1996) or LPS (Bluthé et al., 1992, Long et al., 1990b). Central administration of IL-1 β has similarly been shown to mediate the development of sickness behaviours e.g. decreased social exploration and anorexia (Bluthé et al., 2000b, Bluthé et al., 1994, Harden et al., 2008). Moreover, antagonism of IL-1 β function, by either administering an antagonist (IL-ra) or by knocking out IL-1RI receptors in mice attenuated the development of IL-1 β and LPS-induced sickness behaviours (Bluthé et al., 1995, Bluthé et al., 1997, Bluthé et al., 1992, Ching et al., 2007, Kent et al., 1992).

1.1.2.2 Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF- α) is a potent cytokine that is released by various cells such as macrophages, Kupffer cells, eosinophils, natural killer cells and glial cells (Tracey and Cerami, 1993). TNF- α , which shares the same biological effects as, and is sometimes called cachectin, exists as a membrane-bound prohormone (26 kDa) that can be proteolitically cleaved by the metalloproteinase TNF- α -converting enzyme (TACE) into smaller monomers (17 kDa) in the presence of inflammatory stimuli (Black, 2002, Jue et

al., 1990). Three monomers associate to form a non-covalently bound trimer, which is the predominant active form of TNF- α during systemic inflammation (Tracey and Cerami, 1993). Two membrane-bound receptor types have been identified for TNF- α ; p55 (type I TNFR) and p75 (type II TNFR) (Tartaglia and Goeddel, 1992). Additionally, another soluble TNF receptor (sTNFR), believed to be cleaved from the membrane-bound receptors (Nophar et al., 1990) is also found in serum and exhibits neutralizing or antagonistic effects on TNF- α (Aderka et al., 1992, Engelmann et al., 1989). TNF- α binding is also associated with the activation and translocation of NF- κ B, and the resultant induction of inflammatory gene transcription (Heller and Kronke, 1994).

The role of TNF- α in mediating fever has been the subject of much debate and still remains unresolved because TNF- α appears to exhibit both pyrogenic and cryogenic properties. The pyrogenic effects of TNF- α have been demonstrated in several studies that have shown that: (1) peripheral and central administration of human recombinant TNF- α induced fever in rabbits (Morimoto et al., 1989, Nakamura et al., 1988, Dinarello et al., 1986), and (2) neutralization of TNF- α activity by administration of TNF- α antiserum or TNF- α monoclonal antibodies attenuated the magnitude of the febrile response to LPS and turpentine (Cooper et al., 1994, Luheshi et al., 1997, Nagai et al., 1988). Contrary to these findings, other studies supporting the cryogenic effects of TNF- α have shown that: (1) neutralizing TNF- α activity using TNF- α antiserum or its inhibitory soluble receptor (sTNFR) exacerbated the febrile response to LPS in rats and mice (Kozak et al., 1995, Long et al., 1990a, Töllner et al., 2000a), and (2) TNF- α double receptor knockout mice developed similar or even greater fevers than wild-type mice when challenged with a high dose of LPS (Leon et al., 1997). Consistent with these cryogenic effects, TNF- α has been implicated in mediating hypothermia during sepsis (Derijk and Berkenbosch, 1994, Kozak et al., 1995, Leon, 2004, Töllner et al., 2000a), a regulated response that is believed to improve the chances of survival in life-threatening states of infection (Romanovsky and Szekely, 1998).

Both peripheral and central administration of recombinant human TNF- α was shown to induce depression of social exploration and body mass gain in mice (Bluthe et al., 1994). Similarly, blockage of TNF- α though central administration of sTNFR-binding protein attenuated the depressive effects of LPS in IL-1RI knockout mice (Bluthé et al., 2000b).

These findings tend to support the involvement of TNF- α in mediating sickness behaviours. Contrary to these findings however are reports that TNF- α knockout mice developed similar sickness behaviours to those noted with wild type mice after intraperitoneal LPS administration (Leon et al., 1997). Other studies have similarly shown that blockage of endogenously released TNF- α had no effect on nocturnal activity and minimal effects on LPS-induced anorexia (Töllner et al., 2000a, Harden et al., 2006).

In light of these conflicting findings, it is difficult to consistently explain the mechanisms by which TNF- α induces fever and sickness behaviour. What appears clear from most studies, however, is that TNF- α is released at critical phases during the sickness response and may mediate the sickness response through central rather than peripheral mechanisms.

1.1.2.3 Interleukin-6

Interleukin-6 (IL-6) (initially called interferon β_2 /B cell differentiation factor/B cell stimulation factor) is a glycoprotein (20-30 kDa) secreted by various types of cells including monocytes, fibroblasts, endothelial cells, Kupffer cells, glial cells and neurons (Naka et al., 2002). IL-6 exerts its biological effects by binding to either a membrane-anchored receptor (mIL-6R) or a soluble form of the IL-6 receptor (sIL-6R) obtained from proteolytic cleavage of mIL-6R (Rose-John et al., 2006, Müllberg et al., 1994). Unlike most soluble receptors that compete with their membrane bound counterparts, sIL-6R has been shown to facilitate IL-6 effects on cells that do not express the membrane-bound form (Jones and Rose-John, 2002). The binding of IL-6 to both receptors induces their association with a ubiquitously expressed, membrane-bound signal transducer gp130. The signal transducer gp130 homodimerises and is phosphorylated on its cytoplasmic site by tyrosine kinases of the Janus Kinase (JAK) family. The phosphorylation of gp130 facilitates the docking of signal transducers and activators of transcription (STATs), mainly STAT3 (Heinrich et al., 1998, Heinrich et al., 2003). Subsequent to binding, STAT3 is phosphorylated, dimerises and translocates to the nucleus where it is activated, through further phosphorylation, to regulate transcription of target genes which include pro-inflammatory cytokines, rate limiting enzymes for prostaglandin production as well as regulatory molecules for the JAK/STAT pathway

(Heinrich et al., 1998, Heinrich et al., 2003). IL-6R association with gp130 binding has also been shown to induce activation of other kinase families, such as the Ras kinases, mitogen activated protein kinases (MAPK) and phosphatidylinositol (PI)-3 kinase, with subsequent activation of the positive transcription factor, nuclear factor for IL-6 (NF-IL6) in addition to NF- κ B (Akira et al., 1995). Activation and nuclear translocation of NF-IL6 has been associated with the transcription of inflammatory genes and acute phase proteins during infection (Damm et al., 2011, Schneiders et al., 2015a, Akira et al., 1990).

There is abundant experimental evidence supporting the involvement of IL-6 in mediating fever (Dinarello et al., 1991a, LeMay et al., 1990, Lenczowski et al., 1999, Blatteis et al., 1990, Hübschle et al., 2001, Harden et al., 2008). These studies and more have shown that central and peripheral administration of IL-6 induces fever in different species of experimental animals. Other studies also showed that IL-6 deficient mice were unable to develop fever in response to LPS, IL-1 β , or TNF- α (Chai et al., 1996, Sundgren-Andersson et al., 1998). IL-6 is clearly a key and necessary component of the febrile response during infection because neutralizing circulating IL-6 was shown to completely abolish the development of fever in rats injected with LPS (Harden et al., 2006). Both TNF- α and IL-1 β induce IL-6 release and this cytokine may possibly mediate their pyrogenic effects.

Evidence supporting the involvement of IL-6 in mediating sickness behaviour has been provided by several studies that have shown that central or peripheral administration of IL-6 decreased food intake and motor activity (Plata-Salamán, 1999, Plata-Salamán et al., 1988, Schöbitz et al., 1995, Harden et al., 2008). The deletion of the IL-6 gene was also shown to decrease the depressive effects of LPS on social exploration and food intake in mice (Bluthé et al., 2000a), while blockage of IL-6 using antisera abolished the sickness behaviours associated with LPS administration (Harden et al., 2006, Harden et al., 2011).

Once synthesized and released by immunocompetent cells in the periphery, cytokines serve as part of the communication medium between the immune system and the brain, which ultimately orchestrates fever and sickness behaviour.

1.2 Immune-to-brain communication

As discussed earlier, neural cells, glial cells and endothelial cells of the brain vasculature are some of the cells that express receptors for cytokines. Until a few decades ago, the idea that the mobile scavenging cells of the immune system could communicate with a static, “isolated” central nervous system was unimaginable and heretical; from the perspectives of both the neuroscientists and the immunologists [see Quan and Banks (2007) for review]. However, experimental evidence that has progressively been generated by neuroimmunologists has now shown that the immune system does communicate with the brain and that there are a number of ways through which this communication occurs. The four principal ways (illustrated in Figure 1.2) include: (1) transport of cytokines across the blood brain barrier; (2) entry of cytokines through areas lacking the blood brain barrier; (3) binding of cytokines to receptors on the brain vasculature, and (4) direct stimulation of vagal afferents by cytokines and other molecules in the periphery (Banks et al., 1989, Hashimoto et al., 1991, Matsumura and Kobayashi, 2004). Subsequently, neurons and glial cells in the hypothalamus and other nuclei of the brain are then activated by secondary mediators produced locally e.g. prostaglandin E₂ or through neural signalling. These four principal modes through which the immune system communicates with the brain are discussed further.

1.2.1 Transport of cytokines across the blood brain barrier

The physicochemical characteristics of cytokines make it very difficult for them to traverse the restrictive and protective blood brain barrier (BBB) e.g. they are hydrophilic while the BBB favours transport of lipophilic substances. They are also generally large, charged molecules hence diffusion across the non-polar membranes of the BBB would be too slow, if at all possible. Based on these reasons the possibility that cytokines could cross the BBB was initially excluded (Breder et al., 1988), but subsequently Banks and colleagues provided the first experimental evidence that radiolabelled cytokines administered in the periphery could be transported across the BBB through a specific carrier-mediated transport system (Banks et al., 1989). The studies that followed confirmed the existence of transport systems for cytokines including: IL-1 β , TNF- α , and IL-6. These studies showed that the transport system is highly specific and saturable and

that it could be bidirectional or unidirectional (Banks and Kastin, 1991, Banks et al., 1989, Banks et al., 1994, Banks et al., 1991, Gutierrez et al., 1993, Quan and Banks, 2007).

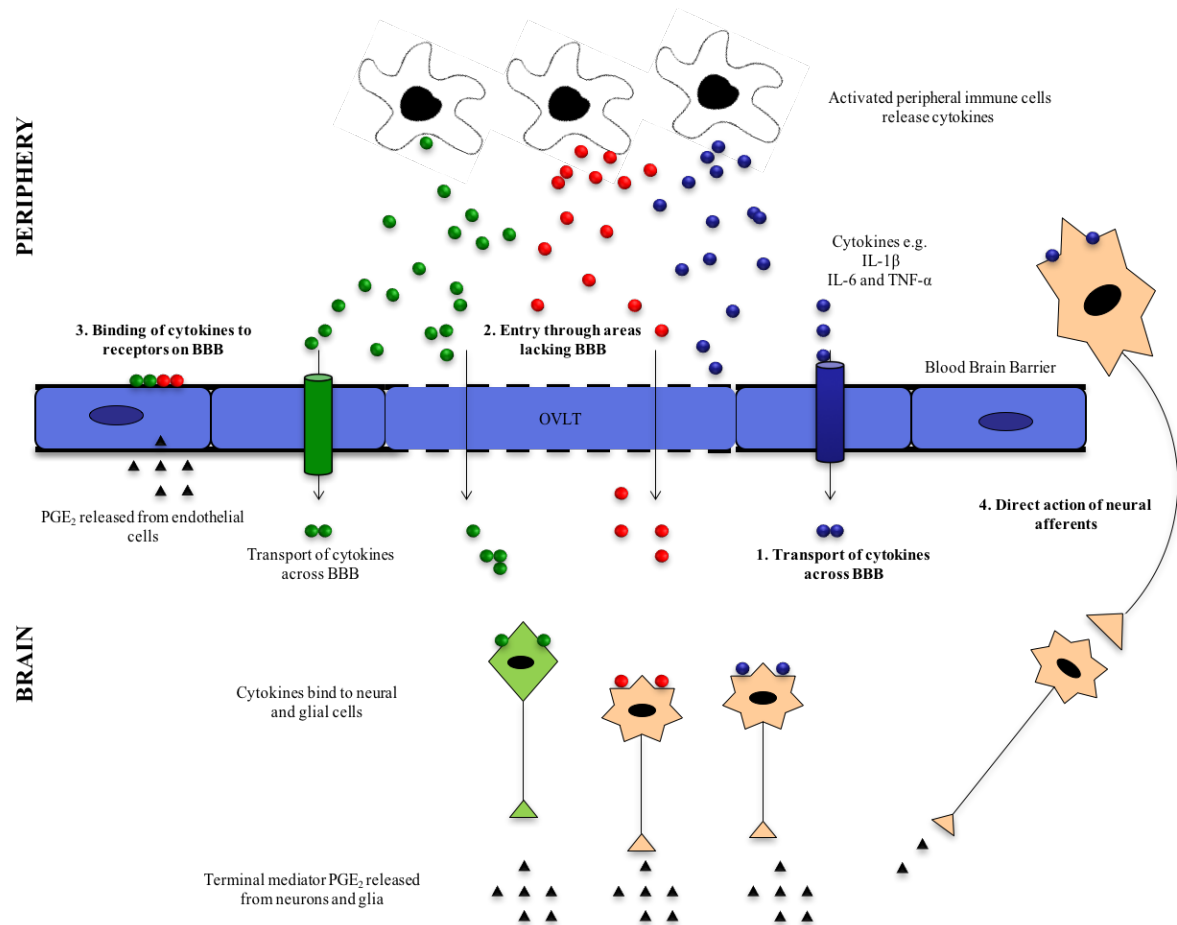


Figure 1.2: Immune-to-brain communication during infection

(1) Cytokines released by immunocompetent cells in the periphery are transported across the blood brain barrier (BBB) through specialized, cytokine transport systems. (2) Cytokines can enter through areas with an incomplete BBB e.g. the vascular organ of the lamina terminalis (OVLT). (3) Cytokines can bind to cytokine receptors expressed on endothelial cells of the BBB and induce the production of prostaglandins within the brain. (4) Peripheral signals transmitted directly to the brain through neural afferents. Brain responses are subsequently mediated by the local production of secondary mediators such as prostaglandin E₂ (PGE₂) as well as by synaptic communications.

(Figure 1.2 was generated by Rachael Dangarembizi from basic principles of immune-to-brain signalling)

1.2.2 Entry of cytokines through circumventricular organs that lack a BBB

In addition to being transported across the blood brain barrier, cytokines may also be transported through circumventricular organs (CVOs); highly vascularized areas of the brain that lack a blood brain barrier and are in close contact with the cerebrovascular system (Roth et al., 2004). The capillaries in the CVOs are fenestrated, thereby permitting drainage of plasma and substances that are usually restricted by the BBB from entering the brain. CVOs were traditionally believed to only take part in homeostatic and endocrine functions, but research has now shown that they are also key components of immune-to-brain communication (Saper and Breder, 1994). These special areas are however delimited from the rest of the brain through the tight junctions of ependymal cells. It has been proposed that cytokines in the circulation most likely enter the perivascular space passively and interact with specific receptors located on the surface of glial cells, perivascular macrophages or endothelial cells. These cells may synthesize small lipophilic secondary mediators e.g. PGE₂ which can diffuse to other brain areas. Alternatively, the CVOs could communicate with other brain areas through neural projections (Roth et al., 2004, Watkins et al., 1995b).

One of the CVOs, the organum vasculosum laminae terminalis or vascular organ of the lamina terminalis (OVLT), has received much attention as a potential port of entry for cytokines during infection and inflammation due to its proximity to and intimate neural connects with the hypothalamus. The OVLT also expresses receptors for IL-1 β , TNF- α and IL-6, further affirming that it is involved in communication between the immune system and the brain. IL-1 conjugated to colloidal gold was injected into rabbits to induce fever, and using electron microscopy the gold particles were observed in the endothelium of the OVLT (Hashimoto et al., 1991). Through immunohistochemistry and real-time polymerase chain reaction (RT-PCR), several studies have shown the activation and nuclear translocation of transcription factors (NF- κ B, STAT3 and NF-IL6) and the subsequent expression of their target genes in the OVLT following a peripheral immune challenge (Damm et al., 2011, Hübschle et al., 2001, Rummel et al., 2011, Rummel et al., 2004).

1.2.3 Binding of cytokines to receptors on the brain vasculature

Besides its obvious role of regulating entry of substances into the brain, the BBB is now recognized as an active component of immune-to-brain communication. Endothelial cells of the brain vasculature express receptors for IL-1 β , TNF- α and IL-6 (Bebo and Linthicum, 1995, Konsman et al., 2004, Vallières and Rivest, 1997) and have also been shown to release various inflammatory molecules including cytokines and prostaglandins (Matsumura and Kobayashi, 2004). To further support the involvement of the BBB in relaying communication from the periphery to the brain during infection, studies have shown a brain specific vascular induction of COX-2 and mPGES during LPS induced fever (Inoue et al., 2002) and that a selective deletion of COX-2 and mPGES in endothelial cells attenuated the inflammatory response and fever associated with a peripheral immune challenge (Wilhelms et al., 2014).

1.2.4 Direct stimulation of vagal afferents

The observations that fever can occur within minutes of an immune challenge and in the absence of detectable levels of cytokines in the circulation have led to the view that a neural pathway may also be involved in immune-to-brain communication (Watkins et al., 1995b, Campisi et al., 2003). In support of the vagus nerve (one of the main nerves that innervate the thoraco-abdominal area) being involved in mediating fever and sickness behaviour are the findings that: (1) vagal neurons express IL-1R mRNA and the prostaglandin E₂ receptor 3 (EP₃) (Ek et al., 1998), and (2) LPS and IL-1 β first produces *cFos* expression in the nucleus of the solitary tract (Brady et al., 1994, Wan et al., 1994), the predominant projection area of vagal afferents, and that this activation can be abrogated by vagotomy (Wan et al., 1994).

Vagotomy in rats and mice has emerged as one of the prominent ways of investigating the role of vagal afferents in mediating fever and sickness and most studies show that vagotomy attenuates the fever and sickness behaviour (Bluthe et al., 1996, Hansen and Krueger, 1997, Sehic and Blatteis, 1996, Watkins et al., 1995a) or at least the early part of it (Romanovsky et al., 1997, Romanovsky, 2004). However, subdiaphragmatic vagotomy attenuated fever and sickness only at low doses of LPS (Romanovsky et al.,

1997) and when injected via the intraperitoneal route. In addition to vagal transfer, cutaneous afferent sensory nerves have also been shown to participate in the induction of fever following subcutaneous administration of LPS (Ross et al., 2000).

Having discussed the four ways by which the immune system communicates with the brain, we will now discuss how communications from the immune system once they get to the brain eventually lead to the development of fever and sickness behaviour.

1.3 PGE₂ synthesis and effects on brain areas that mediate fever and the sickness response

According to the current concepts of the development of fever, prostaglandin E₂ is the ultimate mediator that plays the critical role of stimulating the brain areas that mediate the febrile response (Ivanov and Romanovsky, 2004, Oka, 2004, Nilsberth et al., 2009). The biosynthesis of PGE₂ (summarized in Figure 1.3) begins with the synthesis of arachidonic acid from membrane phospholipids catalysed by phospholipase A₂ (PLA₂) and endocannabinoids catalysed by the recently discovered monoacylglycerol lipase (MAGL) (Sanchez-Alavez et al., 2015). The PLA₂ superfamily is comprised of 19 members that fall under four subclasses. Of these four, only two have been linked with inflammation associated PGE₂ synthesis: cytosolic PLA₂ (cPLA₂) and secretory PLA₂ (sPLA₂). The transcription of cPLA₂ and sPLA₂, particularly that of sPLA₂-IIA, is upregulated in response to inflammatory stimuli (Ivanov and Romanovsky, 2004, Kudo and Murakami, 2002). Recently, an additional PLA-independent, arachidonic acid production pathway was described, in which the endocannabinoid 2-arachidonoylglycerol (2-AG) is hydrolysed by MAGL (Sanchez-Alavez et al., 2015).

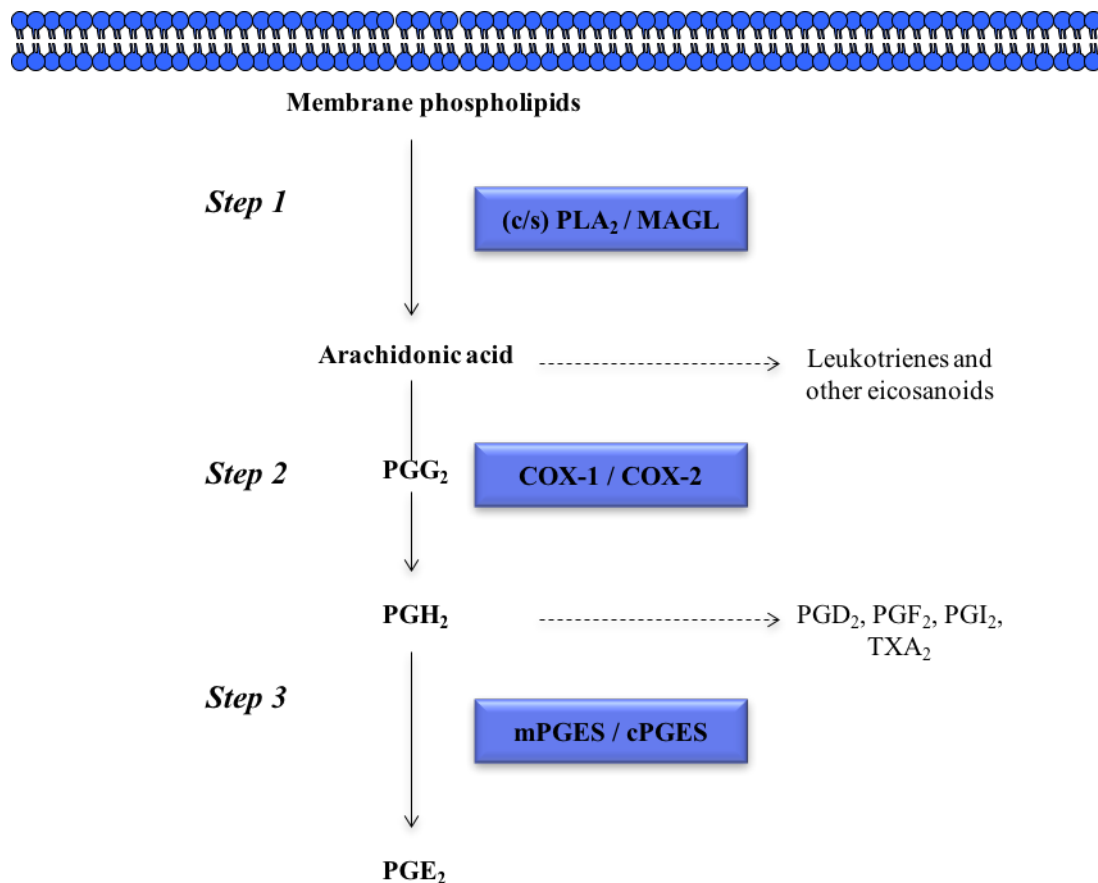


Figure 1.3: Biosynthesis of Prostaglandin (PG) E₂

Step 1: Arachidonic acid synthesis from membrane phospholipids is catalysed by cytosolic or secretory phospholipase A₂ (cPLA₂ or sPLA₂) or monoacylglycerol lipase (MAGL). *Step 2:* PGH₂ synthesis, a two-step reaction catalysed by cyclooxygenases (1 and 2). *Step 3:* PGE₂ synthesis catalysed by microsomal or cytosolic PGE synthase (mPGES or cPGES). Solid arrows represent the main PGE₂ pathway and hashed arrows represent the alternative pathways, which produce other products e.g. leukotrienes, thromboxanes and other prostaglandins.

(Figure 1.3 was modified from Ivanov and Romanovsky (2004)).

Once synthesized, arachidonic acid is converted to prostaglandin H₂ (PGH₂) by cyclooxygenases (COX). Two isozymes of COX have been characterised which share 63% of their sequence and both catalyse the same steps in prostanoid synthesis (Vane et al., 1998). One isomer is constitutively expressed (COX-1) while the expression of the other (COX-2) is induced by inflammatory stimuli (Smith et al., 2000). COX-1 is the house-keeping variant of the enzyme whose physiological functions include, for example, the production of prostaglandins for the maintenance of the integrity of the mucosal epithelium (Vane et al., 1998). COX-1 has also been implicated in the development of LPS-induced hypothermia (Steiner et al., 2009). COX-2 on the other hand is the variant mostly induced by inflammatory molecules such as IL-1 β , IL-6, TNF- α , bacterial

endotoxin, and other pyrogenic molecules (Ivanov et al., 2002). COX-2 has, therefore, received much attention both as a marker for inflammation and as a therapeutic target for alleviating inflammation and fever. Finally, PGH₂ is converted to PGE₂ (and other bioactive products e.g. PGF₂, PGD₂, thromboxane and prostacyclin) by prostaglandin E synthases (PGE synthase). One inducible, membrane-bound form of PGE synthase (microsomal PGES/mPGES), is upregulated, like COX-2, in response to inflammatory stimuli (Ivanov et al., 2002, Murakami et al., 2000, Mancini et al., 2001). Microsomal prostaglandin synthase-1 is an essential and rate-limiting enzyme in PGE₂ synthesis during fever, as shown by the absence of fever in mice lacking mPGES (Engblom et al., 2003, Wilhelms et al., 2014).

Evidence in support of the intermediary role of PGE₂ in fever and some sickness behaviours includes findings that there is a rise in plasma levels of PGE₂ that parallels the development of fever after administration of an exogenous or endogenous pyrogen (Morimoto et al., 1992). Similar to the rise in plasma, others have reported a rise in levels of PGE₂ in the ventricular cerebrospinal fluid (CSF) and in the interstitial fluid around the hypothalamic preoptic area (Bernheim et al., 1980) that follow an immune challenge. Furthermore, inhibition of PGE₂ synthesis blocked fever and sickness behaviour (Scammell et al., 1998) while mice deficient in type 1 and type 3 prostaglandin E receptors did not develop fever after an immune challenge (Oka et al., 2003).

There are four subtypes of prostaglandin E₂ receptors namely; EP₁, EP₂, EP₃ and EP₄ (Sugimoto and Narumiya, 2007). Of the four subtypes, the EP₃ receptor mediates PGE₂-induced fever and sickness behaviour although EP₁ and EP₄ receptors may also play a part (Oka et al., 2003). The EP₃ receptor is expressed in key areas of the brain involved in mediating fever and sickness behaviour (Matsumura et al., 1992, Nakamura et al., 1999, Sugimoto et al., 1994). EP₃ receptors are abundantly expressed in areas that receive immune-related signals from the periphery such as the nucleus of the solitary tract, which receives vagal inputs during infection, and the anterior wall of the third ventricle (OVLT region) which receives humoral signals during infection (Matsumura et al., 1992, Ek et al., 2000). The expression of EP₃ receptors was also detected in brain areas that control sleep and wakefulness, namely the raphe nuclei and the locus coeruleus (LC) (Matsumura et al., 1992, Ek et al., 2000). Additionally, brain areas that mediate pain sensation and suppression have also been shown to express EP₃ receptors and these include the

parabrachial nucleus, thalamus, raphe nuclei, LC and the periaqueductal grey (PAG) (Matsumura et al., 1992, Ek et al., 2000). Lastly, the highest intensity of EP₃ receptor immunoreactivity was reported in areas that mediate thermoregulatory responses namely median preoptic nucleus (MnPO), medial preoptic area (mPOA), the dorsomedial hypothalamus (DMH) and the medullary raphe pallidus (Nakamura et al., 1999). The evidence presented above affirms the importance of EP₃ receptors in mediating PGE₂ induced fever and sickness behaviour. Further affirmation comes from studies done using EP₃ receptor knockout mice, which showed that mice lacking the EP₃ receptors could not develop fever (Lazarus et al., 2007, Oka et al., 2003, Ushikubi et al., 1998).

As mentioned earlier, the strongest expression of EP₃ was detected on the cell bodies and processes of neurons of the MnPO and the mPOA (Nakamura et al., 1999, Nakamura et al., 2000). Most of the EP₃ expressing neurons in the preoptic region produce gamma amino butyric acid (GABA) (Nakamura et al., 2002) and project to the DMH (Nakamura et al., 2005b) and rostral raphe pallidus (rRPa) (Nakamura et al., 2002), the two areas that stimulate sympathetic activation of thermal effectors which generate heat during the febrile response (Nakamura et al., 2004, Nakamura et al., 2009). The effects of PGE₂ on EP₃ receptors in the peri-OVLT area are inhibitory and resemble the effects of microinjecting GABA_A antagonists (bicuculline and gabazine) i.e. increased thermogenesis, tachycardia and cutaneous vasoconstriction (Osaka, 2008). The binding of PGE₂ to EP₃ receptor expressing neurons in the preoptic area is thought to deactivate their tonic inhibition of the downstream sympathoexcitatory neurons in the DMH and rRPa (Nakamura et al., 2009). Disinhibition of the DMH and rRPa in turn stimulates the sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord ultimately leading to an increase in sympathetic activity in the thermoregulatory effector organs for non-shivering thermogenesis such as interscapular brown adipose tissue (BAT) and the rat-tail artery (Nakamura et al., 2004). The MnPO, DMH and rRPa also activate shivering thermogenesis (Nakamura and Morrison, 2011). Microinjection of muscimol, a GABA_A agonist in each of these regions was shown to inhibit cold defensive and febrile shivering (Nakamura and Morrison, 2011). In addition to stimulating sympathetically driven thermoregulatory responses, the DMH projects neural signals to the paraventricular nucleus (PVN), which in turn activates the hypothalamic-pituitary-adrenal (HPA) axis for production of thermogenic glucocorticoids (DiMicco et al., 2006). Ultimately, the rise in temperature during fever is effected through both autonomic and

endocrine thermogenic mechanisms; an increase in brown adipose tissue (BAT) metabolism (Jepson et al., 1988, Morrison, 2003), increased cutaneous vasoconstriction to reduce heat loss (Rathner et al., 2008, Nakamura et al., 2009), increased muscle contraction (shivering) (Nakamura and Morrison, 2011), and a host of other heat conserving behavioural changes which include postural and environmental preferences.

Prostaglandin effects on other brain areas likewise mediate other sickness behaviours for example the binding of PGD₂ to prostaglandin D1 (DP1) receptors activates the nucleus accumbens and the ventrolateral preoptic nucleus which then inhibits the arousal system (tuberomammillary nucleus, dorsal raphe and the LC) [see Saper et al. (2012) for review]. Anorexia may be mediated by PGE₂ activation of the arcuate nucleus and subsequent inhibition of the areas that promote feeding (DMH, PVN, and lateral hypothalamic area) [see (Saper et al., 2012) for review].

In addition to the activity of PGE₂ as a central mediator of fever, several studies have also reported the involvement of other mediators including opioids (Blatteis and Romanovsky, 1994, Blatteis et al., 1991), substance P (Blatteis et al., 1994, Szelenyi et al., 2005) and endothelin-1 (Fabricio et al., 2005).

Having now reviewed the mechanisms underlying the development of the sickness response as a host defence strategy against infection, it is important for us to briefly discuss other biological effects of fever and sickness behaviours that may potentially be undesirable and prompt intervention.

1.4 The maladaptive side of fever and the sickness behaviours

“Humanity has but three great enemies: Fever, famine and war; of these by far the greatest, by far the most terrible, is fever”, was a witty remark from William Osler (Osler, 1896) who voiced his apprehension, shared by many past and present physicians, on dealing with fever and the debilitating nature of sickness in patients with infectious diseases. Fever, often accompanied by other non-specific sickness behaviours, is part of the conserved brain-mediated sickness response that is associated with infection and has been regarded as the hallmark for disease. Writings dating back to the historic times of

Hippocrates, when the foundations of modern day science and medicine were laid, are all consistent in their description of the changes in body temperature and the accompanying sickness symptoms that are associated with disease (Atkins, 1982). The debate of whether or not fever is beneficial to the host began during these ancient times and remains even until today.

A rise in body temperature during infection is believed to create a hostile environment for invading pathogens within which their function is impaired, while at the same time optimizing the function of the host's immune cells (Kluger, 1991). Fever is therefore regarded as a preserved, natural means of fighting pathogens and increasing the chances of survival of the host. In accordance with this tenet, experiments done to demonstrate the importance of fever in host immune defence showed that survival in lizards infected with bacteria was improved by allowing them to develop and defend the febrile state, while increased mortality was observed in infected lizards that were injected with an antipyretic (Kluger et al., 1975, Bernheim and Kluger, 1976). These reports were later qualified by further studies that showed that high body temperatures increase the recruitment and migration of immune cells to strategic locations in the body e.g. lymphoid tissue, where they can effectively fight infections (Evans et al., 2000, Chen et al., 2006). Although these and other studies have demonstrated the benefits of fever in the host's defence against an invading pathogen, the high temperatures and accompanying sickness behaviours that follow infections are generally a great source of discomfort and may even threaten survival in certain vulnerable groups of people. For example, temperatures above 38 °C have been associated with febrile seizures and convulsions in children aged between six months and five years (Knudsen, 2000), although this applies only to a subset of this population. Another example where the risks associated with fever may outweigh its physiological benefits is in patients with cardiovascular and pulmonary diseases; the increased metabolic demands associated with fever may cause inimical cardiopulmonary outcomes (Mackowiak and Plaisacne, 1998). Similarly, fever is detrimental in cases of neurological injury and severe sepsis (Ntaios et al., 2015, Thompson, 2015) and currently, the antipyretic arsenal available to mitigate the cost of fever in these conditions is insufficient (Sultar et al., 2004, Greenberg, 2010). Additionally, with the aid of various antibiotic regimens, the biological value of fever as a defence against microbes may not be as critical as in earlier times and thus the use of antipyretic drugs, such as aspirin, to alleviate the discomfort associated with illness is common practice. Even guidelines of

the World Health Organization (WHO) for the clinical management of uncomplicated self-limiting influenza indicate that “supportive care can be provided as needed, such as antipyretics (e.g. paracetamol or acetaminophen) for fever or pain” (Programme, 2009).

1.5 Antipyretics

The history of the use of substances that reduce fever (antipyretics) dates back to ancient times when natural products were used to treat the fever, pain and sickness that accompanied most diseases. Early Egyptian inscriptions dating back to as early as 1500 BC reported the use of willow tree leaves in the treatment of inflammation and fever. An extract from the Ebers Papyrus reads:

“wound is inflamed...[there is] a concentration of heat; the lips of that wound are reddened and that man is hot in consequence...then you must make cooling substances for him to draw the heat out...leaves of the willow” (Mann, 2000).

Interestingly, the description given in this extract fits that of inflammation and fever, and the recommended remedy forms the basis for use of the modern day pharmacologic antipyretic drug, aspirin. An abridged history of antipyretic drug discovery is summarised in Table 1.1. In the mid-to-late 20th century, the mechanisms underlying the antipyretic properties of antipyretic products were elucidated by Sir John Vane and his colleagues who showed that aspirin and other related antipyretics, collectively known as non-steroidal anti-inflammatory drugs (NSAIDs), are inhibitors of cyclooxygenases and therefore disrupt prostaglandin synthesis (Ferreira et al., 1971, Vane, 1971, Flower and Vane, 1974). Aspirin and related drugs may also attenuate inflammation and fever by other mechanisms which include: (1) inhibition of leukocyte adhesion and migration through endothelial cells (Hofbauer et al., 1998, Kapiotis et al., 1996) (2) inhibition of pro-inflammatory cytokine synthesis through arresting NF- κ B translocation and stabilising I κ B α (Shackelford et al., 1997, Housby et al., 1999, Pierce et al., 1996) and (3) increasing production of endogenous antipyretics and anti-inflammatory mediators such as adenosine (Cronstein et al., 1994).

Table 1.1: Abridged history of antipyretics

Historical period (Dates if available)	Description/Developments	References
<i>Sumerian era</i> (3500 BC)	Sumerian tablets describe the use of willow (<i>Salix spp</i>) leaves for rheumatic fever	(Jack, 1997, Mahdi, 2010)
<i>Early Egyptian Kingdom</i> (circa 1500 BC)	Ebers Papyrus - willow (<i>Salix spp</i>) leaves for treating inflammation, fever and pain.	(Rainsford, 2016) (Mann, 2000)
<i>Hippocratic era</i> (circa 2400 years ago)	Hippocrates – willow bark for pain	(Gross et al., 1948) (Levesque and Lafont, 2000)
<i>Roman Empire</i> (AD 30-60)	Aulus Cornelius Celsus –willow leaves used for relieving pain and inflammation	(Rainsford, 2016)
<i>Early Chinese Kingdoms</i>	Use of <i>Populus alba</i> L and <i>Salix babylonica</i> L for treating rheumatic fever, colds and wounds	(Gross et al., 1948) (Levesque and Lafont, 2000)
<i>Middle ages</i> (circa 1486)	Use of willow bark and leaves for wounds and ulcers.	(Levesque and Lafont, 2000)
1759-1760	Use of <i>Salix</i> genera leaves for treating fevers	(Levesque and Lafont, 2000)
1763	Reverend Edward Stone-first clinical trial of salicylate containing preparations for treating fever	(Stone, 1763)

<i>19th century</i>		
1828-1838	Extraction, isolation, purification of salicylates and salicylic acid	(Hedner and Everts, 1998, Mackowiak, 2000)
1875-early 1890's	Full experimental reports of the antipyretic effects of salicylic acid	(Rainsford, 2016, MacLagan, 1876)
1899	Release of commercial acetylsalicylic acid (aspirin) as an antipyretic drug	
<i>20th century</i>	Continued development of other antipyretic drugs e.g. antifebrin, phenacitin, acetaminophen, fenamates, indomethacin	(Mackowiak, 2000, Rodnan and Benedek, 1970)
	Elucidation of mechanisms of antipyretic drugs	(Vane and Botting, 2003, Vane and Botting, 1997, Vane and Botting, 1998)
<i>Present day</i>	Continued research on developing cheap, safe and effective antipyretics with minimal side effects	

As discussed earlier (see section 1.3), there are two isoforms of COX; the constitutive and physiological COX-1, and the inducible and pathological COX-2. The first NSAIDs used as antipyretics exhibited inhibitory effects on both isoforms and some (e.g. aspirin, indomethacin and ibuprofen) showed a much greater inhibitory effect against COX-1 than COX-2 resulting in undesirable side effects, such as gastric mucosa damage, gastric bleeding and renal damage (Mitchell et al., 1993, Dunn and Zambraski, 1980, Singh, 1998). It appeared that drugs with greater COX-2:COX-1 activity would act as better anti-inflammatory and antipyretic drugs with minimal side effects. Thus around the late 90's the pharmaceutical industry started to produce selective COX-2 inhibitors, such as celecoxib and rofecoxib (Jüni et al., 2002, Masferrer et al., 1994). However, these selective COX-2 inhibitors also exhibit undesirable side effects. By blocking the production of PGI₂, the selective COX-2 inhibitors are thought to disrupt the antagonistic effects of PGI₂ on Thromboxane A₂ (TXA₂), a potent vasoconstrictor and inducer of platelet aggregation (Dogné et al., 2005). A PGI₂/TXA₂ imbalance is associated with an increased risk of acute myocardial infarction, thrombosis, atherosclerosis and other cardiovascular complications (Leval et al., 2004). Apart from the cyclooxygenase related side effects, other antipyretics have also been associated with other undesirable effects. For example, there have been cases of acetaminophen-related hepatotoxicity reported in patients taking acetaminophen with alcohol, in patients taking acetaminophen together with other medications that promote the function of cytochrome P450, as well as in cases of chronic or acute overdose (McClain et al., 1988).

The side effects associated with the use of the conventional NSAIDs have fuelled the quest for cheaper, safer and more effective antipyretic and anti-inflammatory drugs. With hundreds of articles published just over the past decade, research on natural products exhibiting antipyretic and anti-inflammatory effects appears to be an ever-expanding field. Rodents are commonly used as animal models for antipyretic testing.

1.6 *In vivo* protocol commonly used for testing antipyretics

The efficacy of an antipyretic agent is usually determined by measuring the ability of the agent to suppress fever. An accurate determination of a drug's ability to suppress

fever is therefore contingent on an accurate method for measuring body temperature (thermometry).

1.6.1 Choice of thermometry methods

Generally, the choice of a good thermometry method is governed by the purpose of temperature measurement and the nature of the study. For pharmacognostic and pharmacological studies there are a few basic properties that would be desirable when one considers choosing a thermometry method. A good thermometry method should: (1) have a high level of accuracy and sensitivity to changes in temperature and should allow reasonable assessment of the temporal effects of the drug being tested, (2) have minimal artefacts and interference, and (3) cause minimal distress and/or discomfort to both the experimental subjects and the researcher as these may cause errors and inaccurate deductions. To date, the two most commonly used thermometry methods in pharmacognostic and pharmacological studies are biotelemetry and rectal thermometry.

1.6.1.1 Biotelemetry

Biotelemetry is a technique that allows for remote measurement of a number of physiological parameters in free-moving, conscious animals in their natural environment (Kramer et al., 2001). The telemetry system comprises a telemeter, which is surgically implanted into the abdomen of the animal, and sends amplitude modulated radio waves to a receiver that is usually placed underneath the animal's cage (Kramer and Kinter, 2003). The receiver then sends all the data to an in-house data acquisition and processing system, which consolidates the data and provides meaningful output (Kramer and Kinter, 2003). For example, if the physiological parameter being measured is temperature, the frequency of the radio waves sent by the calibrated temperature-sensitive radiotelemeter to the receiver is correlated with temperature and converted to degrees Celsius. Radiotelemetry systems have made significant advances, with current systems able to measure more than 20 physiological parameters which include temperature, locomotor activity, heart rate, blood pressure, blood velocity and respiratory rate (Kramer and Kinter, 2003).

In studies investigating the efficacy of antipyretic drugs, biotelemetry offers the following advantages: (1) an ability to monitor thermal responses round the clock to assess the progression of sickness and the duration of action of the drugs being tested (2) minimal handling and hence reduced influence of method-related confounding artefacts and (3) an ability to measure other important indices of sickness such as reduced locomotor activity (Gegout-Pottie et al., 1999). Studies investigating the postsurgical impact of telemeter implantation on growth, feeding and drinking patterns in rodents, have reported no adverse effects in rats (Leon et al., 2004). Mice have however been noted to show decreases in body weight, locomotion and eating for up to 14 days post-surgery (Baumans et al., 2001). The adverse effects reported in mice may have been related to the small animal body-to-transmitter (B:T) size ratio (Baumans et al., 2001). Current designs of smaller transmitters that have been recently developed may, however, reduce this problem.

The major drawback of using telemetry is the high set-up and maintenance costs and the unavoidable need for surgical implantation of the telemeters (Kramer and Kinter, 2003, Kramer et al., 2001). Due to these limitations, many researchers undertaking pharmacological studies have chosen to use rectal thermometry to measure changes in body temperature.

1.6.1.2 Rectal thermometry

From the 1970s to present day, rectal thermometry has remained the thermometry method of choice in pharmacological studies that investigate temperature responses to various drugs (see Table 1.2).

Table 1.2: Pyrogenic models and thermometry methods used in testing antipyretic drugs from a subset of studies carried out in the periods 1970-1980 and 2015-2016

Anti-inflammatory/Antipyretic Compound	Pyrogen used	Thermometric method	Reference
1970 - 1980			
Aspirin	Brewer's yeast	Rectal	(Loux et al., 1972)
Fenclofenac	Brewer's yeast	Oesophageal	(Atkinson and Leach, 1976)
Δ^9 -tetrahydrocannabinol	Brewer's yeast	Rectal	(Sofia et al., 1973)
Non-steroidal anti-inflammatory drugs (ketoprofen, indomethacin, ibuprofen, phenylbutazone and aspirin)	Lipopolysaccharide	Rectal	(Tachizawa et al., 1977)
Mepacrine	Brewer's yeast	Rectal	(Spagnuolo et al., 1978)
3,4 disubstituted thiophenes	Brewer's yeast	Rectal	(Press and Safir, 1980)
[2-(2, 3-didecanoyloxy)-propyl] 2-acetyloxybenzoate	Brewer's yeast	Rectal	(Carter et al., 1980)
5, 8-dioxygenated isoquinoline-4-carboxylates	Brewer's yeast	Rectal	(Allen Jr and Weiss, 1970)
Substituted pyridines and diazines	Brewer's yeast	Rectal	(Fanshawe et al., 1976)

2-(3'-trifluoromethylthio-anilino)-nicotinic acid	Brewer's yeast	Rectal	(Giudicelli and Najer, 1974)
2-oxo-phenylbutanoic acid	Brewer's yeast	Rectal	(Kigasawa et al., 1979)
Substituted phenylacetic compounds	Brewer's yeast	Rectal	(Diamond and Santora, 1974)
Furan derivatives	Brewer's yeast	Rectal	(D'alo, 1978)
Cyclopenta [b] indole-2-carboxylic acids	Brewer's yeast	Rectal	(Berger and Corraz, 1977)
2-[4-(3-methyl-2-thienyl) phenyl] propionic acid	<i>Mycobacterium butyricum</i>	Rectal	(Kodama et al., 1980)
Indomethacin, aspirin and paracetamol	<i>Shigella dysenteriae</i>	Rectal	(Feldberg et al., 1973)

2015 - 2016

<i>Acacia hockii</i> and <i>Kigelia africana</i>	Turpentine	Rectal	(Kamau et al., 2016)
<i>Kickxia ramosissima</i>	Brewer's yeast	Rectal	(Jan and Khan, 2016)
<i>Delonix regia</i>	Brewer's yeast	Rectal	(Patra et al., 2016)
<i>Artemisia herba-alba</i>	Brewer's yeast	Rectal	(Jaleel et al., 2016)
Qingkailing	Brewer's yeast	Rectal	(Qin et al., 2016)

<i>H. abyssinica</i> Oliv and <i>L. buchananii</i>	Brewer's yeast	Rectal	(Nthiga et al., 2016)
Baicallin	Lipopolysaccharide	Rectal	(Ye et al., 2015)
<i>Jasminum sambac</i>	Brewer's yeast	Rectal	(Sengar et al., 2015)
<i>Stahlianthus involucratus</i>	Brewer's yeast	Rectal	(Pingsusaen et al., 2015)
<i>Ocimum sanctum</i>	Brewer's yeast	Rectal	(Kumar et al., 2015)
<i>Eupatorium chinense</i>	Brewer's yeast	Rectal	(Jiang and Xu, 2015)
<i>Dalbergia saxtalis</i>	Brewer's yeast	Rectal	(Hassan et al., 2015b)
<i>Microcos paniculata</i>	Brewer's yeast	Rectal	(Aziz, 2015)
Silymarin from <i>Silybum marianum</i>	Brewer's yeast	Rectal	(Amin and Arbid, 2015)
<i>Viola yedoensis</i>	Lipopolysaccharide	Rectal	(Ying-yi et al., 2015)
<i>Euphorbia helioscopia</i>	Brewer's yeast	Rectal	(Saleem et al., 2015)
NOSH Sundilac (AVT-18A)	Lipopolysaccharide	Rectal	(Kashfi et al., 2015)
<i>Ixora pavetta</i> Andrews	Brewer's yeast	Rectal	(Mondal et al., 2015)

Of the studies undertaking pharmacological antipyretic testing between 1970 and 1980, only studies that clearly described the method of temperature measurement and their pyrogen and method of inducing fever were included in formulating Table 2. A similar criterion was used to obtain recent studies, but due to increases in the volumes of this type of work in recent years, a comparable number was obtained within 1 year (2015-2016). Comparisons of temperatures measured using rectal thermometry and biotelemetry have yielded conflicting findings; with some studies reporting lower and others higher temperatures measured using rectal thermometry than those measured using abdominally-implanted radiotelemeters (Dallmann et al., 2006, Bae et al., 2007, Dilsaver et al., 1992). The conflicting findings noted with the use of rectal thermometry and biotelemetry could be the result of technical differences, such as the: (1) varying sensitivities of the devices used, (2) differences in thermocouple insertion depths used (the farther away from the liver, the lower the temperatures are likely to be), or (3) stress-related effects associated with the procedure of rectal temperature measurement.

1.6.1.3 Rectal thermometry and stress-induced hyperthermia

The procedure of rectal temperature measurement has been associated with stress-induced physiological changes, which include a rise in body temperature (stress-induced hyperthermia) (Bae et al., 2007, Dallmann et al., 2006, Poole and Stephenson, 1977), an increase in circulating levels of corticosterone (Veening et al., 2004) and inflammatory cytokines (Hale et al., 2003). Stress-induced hyperthermia is a common physiological response exhibited by various species of animals to stressful events, such as handling, restraint (Hashimoto et al., 2001, Parrott and Lloyd, 1995, Shibata and Nagasaka, 1982), exposure to an open field (Butterweck et al., 2003, Grundmann et al., 2006, Kluger et al., 1987, Rowsey et al., 2001), separation, disturbance, injection (Van Der Heyden et al., 1997), exposure to a novel environment and switching of cages (Morimoto et al., 1991, Long et al., 1990c).

Regardless of the stressor, researchers have generally agreed that stress-induced hyperthermia is an acute response, the magnitude of which depends on the nature of the stimulus (Bouwknicht et al., 2007, Vinkers et al., 2008, Parrott and Lloyd, 1995).

Although there are many studies that have reported the occurrence of stress-induced hyperthermia in normothermic rats, very few studies have investigated the development of stress-induced hyperthermia in febrile rats. The few that have, have shown that although prominent in normothermic rats, the hyperthermic response to rectal temperature measurement appears to be muted in febrile rats (Vinkers et al., 2009). During antipyretic drug testing, the efficacy of the antipyretic drug is usually obtained by comparisons made between the temperatures of treated febrile rats and their normothermic counterparts. The induction of stress-induced hyperthermia in normothermic, but not febrile rats could negatively impact the accuracy and reliability of results obtained in these studies.

Although not commonly used for pharmacognostic antipyretic testing there are other alternatives for temperature measurement e.g. a chronic insertion of the probe in a restrained or restricted rat to continuously measure rectal or colonic temperatures. Partial obstruction of the rectum and sustained stress-induced hyperthermia resulting from restraint, handling and possibly the presence of the thermocouple probe have been reportedly associated with this technique (Poole and Stephenson, 1977, Gordon, 1990).

The methods of thermometry described in this section are used to determine thermal responses to administration of a fever-causing agent (pyrogen). Thus, as much as it is important to use an accurate thermometry method, it is equally important to consider the characteristics of the sickness responses induced by one's pyrogen of choice.

1.6.2 Choice of a pyrogen for in vivo antipyretic testing

In pharmacological studies, an experimental pyrogen maybe considered good if it satisfies most or all of the following conditions: (1) a good success rate i.e. an ability to induce fever in most or all of the experimental subjects when administered, (2) an ability to induce a significant increase in temperature which is reasonably uniform and reproducible, (3) an early onset but also a reasonable duration to allow assessment of pharmacological effects, and (4) a reasonable sensitivity to antipyretics. Other factors that affect the choice of a pyrogen may include availability, cost, storage and other cost or logistics-related factors. Although LPS from *Escherichia coli* is a well-characterised

pyrogen for inducing experimental fevers, the pyrogen that is most commonly used in testing pharmacognostic antipyretic drugs is live *Saccharomyces cerevisiae*. In fact, *S. cerevisiae* is recommended for use as an experimental pyrogen in pharmacological antipyretic drug-testing guidelines (Thangaraj, 2016, Vogel, 2002)

1.6.2.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a saprophytic, unicellular, uninucleate, eukaryotic fungus, whose use in the baking (Baker's yeast) and brewing (Brewer's yeast) industry dates back to antiquity. Recent advances in the field of biotechnology have also extended the use of *S. cerevisiae* as a cloning vector for production of mammalian proteins, such as vaccines, interferons, hormones, enzymes and other high value natural products (Kingsman et al., 1987, Krivoruchko and Nielsen, 2015). *S. cerevisiae* is also popularly used as a probiotic and for treating *Clostridium difficile*-related diarrhoea (Segarra-Newnham, 2007).

The yeast cell is comprised of a protoplast that is surrounded by a cell wall made up of β -1,3 and β -1,6 glucans (30-50%), mannoproteins (40%), chitins, lipids and other proteins (Klis et al., 2006, Lipke and Ovalle, 1998). The glycoprotein and polysaccharides in yeast cell walls are covalently linked to form macromolecular complexes and the glycosylations and linkage patterns vary from species to species and within species (Klis et al., 2006, Lipke and Ovalle, 1998). Generally, *S. cerevisiae* has been regarded as a safe commensal. Recently however, virulent strains of yeast have been isolated and implicated in invasive fungal infections, which include vaginitis (Sobel et al., 1993) and systemic infections predominantly in immunocompromised patients and transplant recipients (Popiel et al., 2015, Silva et al., 2011, Aucott et al., 1990, Cimolai et al., 1987). These and other studies therefore show that contrary to the common belief that *S. cerevisiae* is safe and non-pathogenic, *S. cerevisiae* is an emerging opportunistic pathogen, which has the ability to invade, proliferate and cause illness (Mccusker, 2006).

The pathogenicity of Brewer's yeast is due to the presence of the distinctive highly glycosylated proteins and polysaccharides on the surface of the yeast's cell wall that are recognized as PAMPs by the innate immune system (Levitz, 2004). The recognition of β -1,3 glucans by the CLR Dectin-1 and β -1,6 glucans by complement receptor 3 (CR3)

is known to trigger an immune response (Romani, 2011, Levitz, 2010). Dectin-2, langerin and the mannose receptor recognise mannans, while mincle receptor recognizes chitin (Romani, 2011, Levitz, 2010). O-linked mannans and other yeast cell components such as DNA and RNA activate TLRs (mainly TLR2, TLR6 and TLR9) and trigger inflammation and subsequently sickness (Romani, 2011).

Despite its extensive use in antipyretic screening studies over the last 40-50 years (see Table 1.2), there are very few studies, in refereed literature, that have fully characterised the febrile response to Brewer's yeast injection. Of the few studies that have been done, rectal thermometry has been used but as described earlier in section 1.6.1.3 of this thesis, rectal temperature measurement has methodological limitations. In addition to inducing hyperthermia, rectal temperature measurement is restricted to 30-60 min intervals and therefore does not allow the continuous temperature recordings much needed to fully describe thermal responses. Moreover, because of its cumbersome and labour-intensive nature, rectal thermometry does not easily lend itself for temperature measurements to continue over long periods of time. As a result of these methodological limitations, the exact characteristics of fever induced by the standard doses of Brewer's yeast used in antipyretic screening studies are largely unknown. Knowing the characteristics of a fever, such as latency, amplitude and duration, is critical when testing antipyretics as the drug being tested must not be administered for example during the falling phase when temperatures are naturally decreasing. Without the data on the characteristics of the febrile response to common doses of Brewer's yeast used in antipyretic testing, it is difficult to determine at which point along the course of the fever the pharmacological manipulations are being done. Thus, the reliability of the observed antipyretic effects is compromised.

In addition to the lack of data describing the characteristics of the febrile response, there is also a paucity of data describing the molecular mechanisms subserving fever and sickness behaviour responses to Brewer's yeast administration. From some of the work that has been done it appears that Brewer's yeast may induce an inflammatory response similar to that described earlier following LPS administration. For example, some studies have shown that administration of COX-inhibitors abrogates Brewer's yeast-induced fever (Loux et al., 1972, Opas et al., 1987), suggesting that Brewer's yeast-induced fever is prostaglandin dependent and cyclooxygenases may play an active role. From studies

done on other pathogenic fungi, we already know that activation of TLR2/TLR6 and Dectin-1 leads to downstream activation of NF- κ B (Saijo and Iwakura, 2011). Thus, Brewer's yeast may activate NF- κ B signalling pathways which trigger the production of pro-inflammatory cytokines (Gantner et al., 2003). To support the possible involvement of pro-inflammatory cytokines in mediating Brewer's yeast-induced fever, a study done in rats showed that intraperitoneal (i.p.) injection of Brewer's yeast was associated with increased concentrations of TNF- α and IL-1 β in peritoneal fluid (Ferreira et al., 2012). Similarly, injection of mannan components isolated from the cell wall of Brewer's yeast increased serum levels of TNF- α in rats (Ataöglu et al., 2000). *In vitro* studies done using macrophages have also shown that NF- κ B activation and TNF- α production are increased in response to stimulation with Brewer's yeast, or other yeast cell wall components (Brown et al., 2003, Gantner et al., 2003, Young et al., 2001). Although these studies show the possible involvement of some inflammatory molecules in Brewer's yeast-induced fever, they do not provide a full spatiotemporal picture of the activation of the mediators in relation to changes in temperatures and other sickness behaviours.

The standard doses of whole *S. cerevisiae* used to induce fever during antipyretic drug testing are very high, ranging between 2 - 4 g/kg (Hassan et al., 2015b, Josa et al., 2001, Pingsusaen et al., 2015). The subcutaneous route is the recommended and commonly used route of administration of Brewer's yeast most likely because it mimics a localized peripheral infection, is less invasive and is associated with a high success rate unlike that noted via the intraperitoneal route. However, subcutaneous administration of high doses of Brewer's yeast during injection of Brewer's yeast at high doses has been shown to induce the development of inflammatory abscesses and granulomas (Todd, 1986). There remains therefore the need to develop other effective pyrogens with less side-effects. One potential alternative is zymosan, a cell wall moiety of Brewer's yeast.

1.6.2.2 Zymosan

Zymosan is a purified cell wall product of *S. cerevisiae* that is commonly used as a phlogistic agent in animal models of arthritis (Keystone et al., 1977), peritonitis, pleurisy (Utsunomiya et al., 1998), oedema, pain and general inflammation. Although zymosan is

mainly used in studies investigating the mechanisms of local inflammation, this moiety of *S. cerevisiae* may be a good candidate for simulating fungal infections and testing antipyretic agents. Zymosan primarily contains β -glucans and mannans that activate the alternative complement pathways and immunocompetent cells such as macrophages, neutrophils (Doherty et al., 1985), natural killer cells (Duan et al., 1994), and mast cells. Like live yeast, zymosan initiates the inflammatory response by binding to TLR2/TLR6 and Dectin-1 (Brown et al., 2003, Keystone et al., 1977). Mouse macrophages stimulated by zymosan showed redistribution of TLR2 from the cell surface to the phagosome (Underhill et al., 1999). Similarly, stimulation of macrophages with radiolabelled zymosan showed that the binding of zymosan to Dectin-1 requires the cooperative action of TLR2 and its associated immunoreceptor tyrosine-based activation motifs (Brown et al., 2003) to initiate an inflammatory response. Zymosan activates the NF- κ B and JAK/STAT inflammatory signalling pathways (de Hooge et al., 2004a) and is associated with an increase in the pro-inflammatory cytokines TNF- α (Utsunomiya et al., 1998, Asmuth et al., 1990a), IL-1 β (Fantuzzi et al., 1997) and IL-6 (Utsunomiya et al., 1998, Asmuth et al., 1990a, Bastos-Pereira et al., 2014). Additionally, zymosan administration substantially increases the production of PGE₂ (Doherty et al., 1985), eicosanoids (Rao et al., 1994), chemoattractants (Utsunomiya et al., 1998) and the migration of neutrophils to the site on infection (Ajuebor et al., 1998). Stimulation of primary neuroglial cultures, obtained from the OVLT and the MnPO, with zymosan has also been shown to increase TNF- α and IL-6 (Bastos-Pereira et al., 2014). The findings from the studies discussed above provide evidence that zymosan is a PAMP that elicits an immune response and activates similar inflammatory mediators and immune-to-brain communication pathways to those mentioned previously with LPS administration (see section 1.1.2).

Studies characterising the inflammatory response to zymosan-induced arthritis in rats, (Gegout et al., 1994) have observed a febrile response, albeit short-lived (4h), following intra-articular injection of 4 mg/kg zymosan. Moreover, intraperitoneal injection of 10 mg/kg zymosan in rats also induced a short-lived (4 h) fever of moderate magnitude (1.2 °C), which was also accompanied by reduced food intake and motor activity on the first day after injection (Hubschle et al., 2007, Naoi et al., 2006). The doses of zymosan used in the above studies to induce arthritis and peritonitis induced very low-to-moderate amplitude, short-duration fevers that may not be desirable for screening antipyretics.

However, intraperitoneal injection of greater doses of zymosan (500 mg/kg) resulted in sepsis, shock and increased mortality in rats (Fantuzzi et al., 1997). Administration of moderate-to-high doses of zymosan could however induce high magnitude, long duration fevers that are desirable for evaluating pharmacological agents.

1.6.3 The incorporation of sickness behaviours during pharmacognostic antipyretic drug testing

As discussed earlier, fever is often accompanied by other cytokine-mediated sickness behaviours, such as lethargy, somnolence, anorexia, general body aches and depression-like behaviour. However, current guidelines for testing antipyretic drugs only focus on the efficacy of test substances in reducing temperature (Thangaraj, 2016) possibly based on an assumption that fever and sickness behaviours are mediated largely by common pathways; hence drugs that are effective in attenuating fever are most likely capable of suppressing sickness behaviours. Research has shown, however, that although both fever and sickness behaviours are cytokine-mediated responses to infection, the mechanisms underlying their development may be different (Harden et al., 2011, Hübschle et al., 2006). During illness, sickness behaviours tend to last long after fever has resolved and from a clinical point of view, it is these prolonged sickness behaviours that are more debilitating and uncomfortable to the patient compared to the slight rise in temperature they may experience during fever (Bower et al., 2002, Hewlett et al., 2005). Thus it is important to test the efficacy of pharmacognostic drugs not only on the thermal response, but so too on the non-thermal responses to infection.

1.7 Thesis Aims

There is a resurgent interest in the search for safe and effective antipyretic drugs to replace or complement the conventional NSAIDs, which have various side effects. Researchers and pharmacological companies are shifting their focus to natural products with possible anti-inflammatory and antipyretic effects. In line with this shift, the field of pharmacognosy has grown considerably over the past few decades. **The general aim** of the research described in this thesis is to characterise and validate the standard models of

inducing fever and methods of temperature measurement used in pharmacognostic antipyretic drug screening studies.

Despite technological advancement and the introduction of computer-based thermometry methods that allow remote, real-time measurements of various physiological parameters in free moving rats, most studies still use rectal thermometry for monitoring temperature changes during antipyretic drug testing (shown by the frequency of use of rectal thermometry in a sample of studies given in Table 1.2). Research has shown that the handling and restraint associated with rectal temperature measurement is associated with stress-related physiological changes including hyperthermia. Stress-induced hyperthermia may affect the accuracy and reliability of thermal responses observed during antipyretic drug testing. **The aim of the experiments described in Chapter 2 of this thesis was therefore to characterise the stress-induced hyperthermia associated with rectal temperature measurement in normothermic and febrile rats.** Fever was induced by subcutaneous injection of Brewer's yeast and changes in core body temperature after rectal temperature measurement were monitored using remote-sensing, thermosensitive telemeters implanted in the abdomen of each rat. Because some studies pre-expose experimental animals to the procedure of rectal temperature measurement for a prescribed period of time prior to the experiments, I also sought to investigate if the standard prescribed method of habituation blunts or reduces the magnitude of the stress-induced hyperthermia. I therefore pre-exposed some rats to the procedure of rectal temperature measurement and compared their thermal response with that of rats naive to the procedure. Lastly, to verify the accuracy of rectal thermometry in measuring core body temperature, I compared the temperatures measured using rectal thermocouple probes with temperatures measured using intra abdominally implanted radiotelemeters.

Having reviewed the literature describing the recommended Brewer's yeast model for inducing fever in antipyretic drug testing, it became apparent that there is little data describing the characteristics of the febrile response induced by administration of the standard doses of Brewer's yeast used in antipyretic drug testing. Without knowledge of the fever characteristics, it is difficult to determine the best time for pharmacological manipulations and drug testing to reliably conclude if the drug indeed exhibits the desired effects. **The first aim of the experiments described in Chapter 3 was therefore to characterise the febrile response and sickness behaviours associated with**

subcutaneous injection of the standard doses of Brewer's yeast used in antipyretic drug testing. Using biotelemetry, I characterised the latency, time to peak, amplitude and duration of the febrile response to subcutaneous injection of Brewer's yeast. Previous studies done mainly using LPS as a pyrogen have shown that in addition to fever, rats also show signs of sickness e.g. lethargy, anorexia and growth-stunting. In addition to fever, these behavioural responses are good indices of sickness and may be useful for assessing the pharmacological effects of drugs. Thus, I also characterised the development of sickness behaviours after subcutaneous injection of Brewer's yeast. I found for the first time that the standard doses of Brewer's yeast used for inducing fever during antipyretic drug testing also induce lethargy, anorexia and growth stunting.

The inflammatory mechanisms accompanying the fever and sickness behaviour associated with Brewer's yeast infection have not been fully elucidated. As a result, of the hundreds of studies reporting positive antipyretic effects from natural products, very few proffer any mechanistic explanations and are limited to merely screening for the presence or absence of antipyretic activity with no knowledge of cellular targets or potential antipyretic mechanisms of action. The progression of newly discovered antipyretic natural products might be limited by a lack of data on the inflammatory pathways activated in the pyrogenic model used for testing them. **The second aim of the experiments described in Chapter 3 of this thesis was to elucidate the spatiotemporal activation of key inflammatory mediators and signalling pathways during Brewer's yeast-induced fever and sickness behaviour.** Using biological assays based on the growth promoting effects and cytotoxic effects of cytokines, I measured the circulating levels of IL-6 and TNF- α at four time points corresponding to the early, peak, plateau and falling phase of Brewer's yeast-induced fever. To measure neuroinflammation, I used immunohistochemical staining and examined the activation and translocation of transcription factors (STAT3, NF- κ B and NF-IL6) in the OVLT. I also measured the expression of their target genes in the hypothalamus using Real Time-Polymerase Chain Reaction at each of the four phases of fever described above. The methods used for measuring neuroinflammation in my experiments are well established methods that have been successfully used in other studies to investigate neuroinflammation induced by LPS (Damm et al., 2011, Rummel et al., 2011). Using the above-described methods I managed

to show, for the first time, the spatiotemporal patterns of activation of inflammatory mediators during Brewer's yeast-induced fever, anorexia and lethargy.

The administration of Brewer's yeast elicited fever, sickness behaviour and inflammatory mediators and therefore qualifies as a valid model for testing antipyretic drugs. Although Brewer's yeast was noted to be a highly effective pyrogen, I observed that the standard doses and volumes used during antipyretic drug testing are associated with the development of an abscess, pain and suffering/discomfort in the rats. According to the animal ethics principle of refinement, procedures and techniques used for collecting data on experimental animals should cause minimal (if any) suffering and pain (Fenwick et al., 2009). I tested a dose of Brewer's yeast lower than the standard doses used, but the inflammatory response noted with the lower dose was short-lived and therefore undesirable for the purposes of testing antipyretic drugs. However, as reviewed earlier in this chapter, zymosan, a cell wall product prepared from Brewer's yeast, is commonly used as an inflammatory agent for inducing arthritis, peritonitis and pleurisy and may be a potential alternative for inducing fever during antipyretic drug testing. **The aim of the experiments described in Chapter 4 of this thesis was therefore to characterise the fever, sickness behaviour and inflammatory mechanisms associated with subcutaneous injection of moderate doses of zymosan in rats.** I found that, like Brewer's yeast, zymosan induces fever and sickness behaviour through the activation of peripheral and neuroinflammatory mediators. The spatiotemporal characteristics of zymosan-induced neuroinflammatory mediator activation was in most cases very similar to that noted with Brewer's yeast.

1.8 References

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CHAPTER TWO

Thermometry methods used during antipyretic testing: Limitations of using rectal thermometry

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2.1 Introduction

Rectal temperature recorded using a thermocouple probe inserted into the rectum and abdominal temperature recorded using a radiotelemeter implanted into the abdomen potentially yield comparable values with regard to core body temperature (Dilsaver et al., 1992). However, one major disadvantage associated with the measurement of rectal temperature in laboratory animals is that the procedure of handling and inserting the thermocouple probe is stressful (Veening et al., 2004). The stress initiated by handling and inserting the thermocouple probe into the rectum leads to a number of physiological changes, which include an increase in body temperature (stress-induced hyperthermia) (Bae et al., 2007, Dallmann et al., 2006) as well as an increase in neuroendocrine hormones and cytokines (Hale et al., 2003, Veening et al., 2004). While the stress-induced hyperthermic response that follows rectal temperature measurement is commonly reported in normothermic rats, it appears to be muted or masked in rats with a fever (Vinkers et al., 2009).

Despite reports of the procedure causing stress-induced hyperthermia, rectal thermometry remains a common technique for measuring antipyresis in rodents in pharmacological, especially ethnopharmacological, studies. Body temperature is the key physiological indicator used to determine the efficacy of the antipyretic pharmacological agents and usually conclusions are drawn from comparisons between the temperatures of treated febrile rats and their normothermic counterparts. Because the technique used for measuring temperature in these studies appears to cause a prominent increase in body temperature in normothermic, but not febrile, rats, it is important to examine the characteristics of this hyperthermia and establish whether the use of rectal thermometry can impact the accuracy and validity of results.

In an attempt to reduce the stress-induced physiological changes associated with rectal temperature measurement, laboratory animals are often habituated to the procedure by subjecting them to the procedure of rectal temperature measurement for two-to-three days before the start of the experiment (Souza et al., 2002, Tomazetti et al., 2005). Whether this protocol indeed habituates the animals to the procedure by blunting the stress-induced hyperthermia remains unclear.

Thus, using a standardized technique for measuring rectal temperature, this study was carried out to investigate the effects of: (1) rectal temperature measurement on temperature responses of normothermic and febrile rats, and (2) commonly used habituation protocols at attenuating the hyperthermic response associated with the procedure of rectal temperature measurement. I used rats that had either been subjected to rectal temperature measurements before the experiment (pre-exposed), or rats that were naive to rectal temperature measurement and administered either saline or a pyrogenic dose of Brewer's yeast to induce fever. During the plateau phase of the fever rectal temperatures were repeatedly measured at hourly intervals in both normothermic and febrile rats while monitoring abdominal temperature changes using intra-abdominally-implanted radiotelemeters. Results obtained in this study show that the procedure of rectal temperature measurement produced a robust, consistent hyperthermic response in normothermic rats, but not in rats with a fever induced by subcutaneous administration of Brewer's yeast. Pre-exposing normothermic rats to rectal temperature measurements on two successive days before the experiment did not affect the magnitude or the duration of the hyperthermic response induced by rectal temperature measurements.

2.2 Materials and methods

2.2.1 Animals

Male Sprague-Dawley rats were purchased from the National Health Laboratory Services (Johannesburg, South Africa) and housed in the Central Animal Service Unit of the University of the Witwatersrand, Johannesburg. Rats were housed individually in acrylic cages lined with wood shavings in a temperature-controlled room (22 ± 2 °C) on a 12h:12h light/dark cycle (lights on at 06:00 clock time). Rats were allowed to acclimate to their environment for seven days before surgery. The rats had access to rodent pellets and water *ad libitum*. On the day of injection of the pyrogen, rats had a body mass of between 250 and 300 g. All experiments were performed in accordance with the regulations of the Animal Ethics Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Screening Committee (ethics clearance number 2014/10/C; see Appendix).

2.2.2 *Abdominal temperature*

Abdominal temperature was recorded by remote biotelemetry using temperature-sensitive radiotelemeters (TA10TA-F40, Data Sciences, St Paul, MN, USA), which had been implanted intra-abdominally. Rats were anaesthetized with 80 mg/kg ketamine hydrochloride (Anaket-V, Bayer, South Africa) and 20 mg/kg xylazine (Chanazine, Bayer, South Africa). After surgery, the rats received a single intramuscular injection of an analgesic, buprenorphine (Temgesic®, 0.05 mg/kg Schering-Plough, Johannesburg, South Africa) and were given seven days to recover from the surgery before the commencement of the experiments outlined below. Transmitter output frequency (Hz) was monitored at one-minute intervals by a receiver plate (RPC-1, Data Sciences, St Paul, MN, USA) placed beneath the cage of each rat. The frequency received by each plate was fed into a peripheral processor connected to a personal computer and converted to degrees Celsius. The radiotelemeters were calibrated individually by the manufacturer (Data Sciences, St Paul, MN, USA), in a water bath at 35 °C and 39 °C, to an accuracy of 0.1 °C.

2.2.3 *Rectal temperature*

Rectal temperature measurements were obtained by gently inserting a lubricated thermocouple probe (Type T copper/constantan thermocouple probe, external diameter 2 mm) 40 mm into the rectum of each rat. An insertion depth of 40 mm has been used successfully for determining core body temperature in rats (Bae et al., 2007, Kiyatkin and Brown, 2005, Kollmar et al., 2007). The polyvinyl chloride-insulated thermocouple probe was calibrated against a high-accuracy thermometer (Quat 100, Heraeus, Hanau, Germany) in an insulated water bath across a range of temperatures (35, 36, 37, 38, 39 and 40 °C) to an accuracy of 0.1 °C. To measure rectal temperature, the rat was placed with all four paws on a smooth, solid surface and gently held in place by a hand positioned over the back, while the tail was lifted and the thermocouple probe was inserted. It took time (30 – 120 s) to settle the rats and position them for the insertion of the thermocouple probe into the rectum. Once inserted the probe remained in the rectum for 40 s to allow temperatures to stabilise (Bae et al., 2007). All rectal temperature measurements were performed by one person (Rachael Dangarembizi).

2.2.4 Pyrogen

Brewer's yeast (Sigma Aldrich, St Louis, USA: Lot# SLBC 5519V) was reconstituted in sterile, non-pyrogenic 0.9 % saline (Sabax, Johannesburg, South Africa) to a final concentration of 20 % (w/v). The yeast solution was injected subcutaneously (s.c.) in the dorsum of the neck at a volume of 20 ml/kg (equivalent to 4000 mg/kg) (Hassan et al., 2015a, Keri et al., 2010).

2.2.5 Experimental procedures

To determine if pre-exposure to the procedure of rectal temperature measurement reduced the magnitude of stress-induced hyperthermia, rectal temperatures were measured once a day (1-2 hours before the onset of the dark phase), for two days before the start of the experiment. These rats were said to be “pre-exposed”, while the other rats that received their first rectal temperature measurement only on the day of the experiment were called “naive”. Pre-exposed and naive rats received a s.c. injection of either Brewer's yeast or saline. As additional controls, another group of twelve rats received a s.c. injection of Brewer's yeast, but without subsequent rectal temperature measurements. In an attempt to reduce the number of animals used for the study I chose not to include an additional group of rats that received saline followed by no rectal temperature measurements (referred to in Figure 2.1 and 2.3B as “no rectal probe”), but rather to use abdominal temperature recordings from pre-intervention days when the rats were left undisturbed in their home cages. The final experimental groups are summarized in Table 2.1.

Injections were administered at 16:00, two hours before the onset of the dark phase. Pharmacological antipyretic testing guidelines in rats recommend that the test compound or the standard drug be administered 18 hours after injection of the pyrogen and rectal temperatures be recorded at regular time intervals of 30-60 minutes for five hours thereafter (Thangaraj, 2016, Vogel, 2002). I therefore started all rectal temperature measurements 18 hours after injection of Brewer's yeast and rectal temperature measurements were taken once every hour, over a five-hour period (10:00-15:00). Following each rectal temperature measurement, the rats were returned to their home cages.

Table 2.1: Summary of experimental interventions and sample size

Intervention	Sample size
Rectal probe pre-exposed + Brewer's yeast	9 (2 rats excluded in final analysis)
Rectal probe naive + Brewer's yeast	9 (1 rat excluded in final analysis)
Rectal probe pre-exposed + Saline [#]	8
Rectal probe naive + Saline [#]	9 (1 rat excluded in final analysis)
No rectal probe + Brewer's yeast (6 of the 12 rats were used for calculating the "baseline" average abdominal temperatures of febrile rats as explained in the data analysis section).	12

[#] In an attempt to reduce the number of animals used for the study I chose not to include an additional group of rats that received saline followed by no rectal temperature measurements

2.2.6 Data analysis

All data were analysed using GraphPad Prism software (version 7.0, GraphPad Software Inc, USA). Statistical significance was accepted for $P < 0.05$. To analyse the fever induced by s.c. administration of Brewer's yeast, the original one-minute abdominal temperature recordings were averaged over two hours and the two-hourly means compared by repeated measures two-way analysis of variance (ANOVA) with intervention and time as main effects. Tukey's *post hoc* test was used to detect differences between groups when the ANOVA detected significant main effects or interactions.

Pearson's correlation analyses were performed to calculate the correlation coefficient (r) between rectal temperatures recorded using a thermocouple probe and abdominal temperatures recorded using a radiotelemeter. A Bland-Altman analysis (Bland and Altman, 1986) was performed to determine the level of agreement between the two methods of temperature measurement.

As mentioned earlier, a group of rats ($n = 12$) received a s.c. injection of Brewer's yeast, but without subsequent rectal temperature measurements. Six of these rats were used to calculate the "baseline" average abdominal temperatures of febrile rats 18 – 23 hours after injection of Brewer's yeast, i.e. during the five-hour experimental period (10:00 – 15:00). To determine the effect of rectal temperature measurements on the abdominal temperature of febrile rats, I calculated the difference between the abdominal temperatures of rats having or not having rectal temperature measurements and the baseline average abdominal temperatures calculated as outlined above. Rats were excluded from data analysis when temperature responses differed by more than twice the standard deviation of the group mean ($n = 2$ Pre-exposed + Brewer's yeast; $n = 1$ Naive + Brewer's yeast).

To determine the effect of rectal temperature measurements on the abdominal temperature of normothermic rats over the five-hour experimental period (10:00 – 15:00), I calculated the difference between the abdominal temperatures of a rat having rectal temperature measurements following injection of saline or not having rectal temperature measurements from the average abdominal temperature of the rat when left undisturbed at exactly the same time of the day, on three previous days. For statistical purposes, the one-minute changes in abdominal temperature values, calculated as outlined above, were averaged over five minutes for the five-hour experimental period (10:00 – 15:00). The five-minutes means were compared by repeated measures two-way ANOVA with intervention and time as main effects. Tukey's *post hoc* test was used to detect differences between groups when the ANOVA detected significant main effects or interactions. Rats were excluded from data analysis when temperature responses differed by more than twice the standard deviation of the group mean ($n = 1$ Naive + Saline).

The maximum change in abdominal temperature, after each rectal temperature measurement in pre-exposed and naive rats that received saline, was regressed linearly against the number of rectal temperature measurements for each rat to determine if normothermic rats habituate to five repeated measurements of rectal temperatures spaced an hour apart.

2.3 Results

Figure 2.1 shows that rats injected s.c. with Brewer's yeast initially had a significant decrease in abdominal temperature (36.81 ± 0.35 °C) two hours after injection compared to rats injected with saline (37.56 ± 0.23 °C). Following the brief period of hypothermia, the abdominal temperature of rats injected with Brewer's yeast started to increase and peaked at 39.02 ± 0.3 °C eight hours after injection and remained significantly elevated for 72 hours compared to rats injected with saline (main effects of time ($F_{(36, 1692)} = 212.8$, $P < 0.0001$), treatment ($F_{(5, 47)} = 149$, $P < 0.0001$) and their interaction ($F_{(180, 1692)} = 29.71$, $P < 0.0001$)).

The difference in the body temperature between rats injected with Brewer's yeast and rats injected with saline represents the amplitude of the fever. Greater fever amplitudes were noted at 18 hours after injection (the time at which fever is usually assessed in pharmacological antipyretic screening studies) with temperature values obtained from radiotelemeters implanted in the abdomen (mean difference 1.79 °C and 95% confidence intervals 1.56 and 2.03 °C), than with temperature values obtained from thermocouple probes inserted into the rectum (mean difference 1.56 °C and 95% confidence intervals 1.31 and 1.8 °C).

The rectal temperature and abdominal temperature values measured at 18 hours after injection were significantly correlated in rats that received saline ($r = 0.79$, $P = 0.0003$) and in rats that received Brewer's yeast ($r = 0.64$, $P = 0.01$).

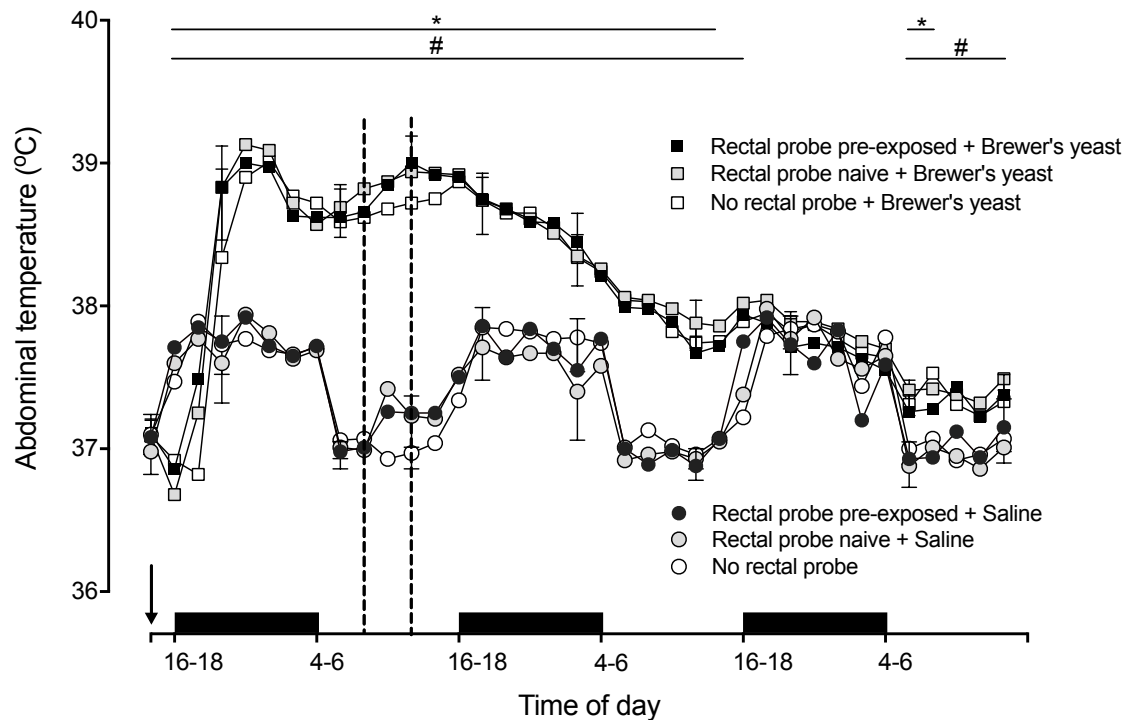


Figure 2.1: Abdominal temperature (mean and SD) of rats injected subcutaneously with saline or 4 g/kg Brewer's yeast in saline. Pre-exposed rats had rectal temperature measurements taken once a day on the two days before receiving an injection of Brewer's yeast (n=7) or saline (n=8). Naive rats received the first rectal temperature measurement 18 hours after receiving injections of either Brewer's yeast (n=8) or saline (n=8). The arrow indicates the time of injection and dashed lines indicate the period during which rectal temperature measurements were taken (10:00-15:00 clock time on day 1 post-injection). The black bars represent night time. Brewer's yeast and no rectal probe represent rats that received an injection but no rectal temperature measurements (n = 6). No rectal probe represents the abdominal temperature when the rats that received saline were left undisturbed in their home cages; i.e. before exposure to rectal temperature measurements started. As there were no significant differences in the temperature profiles obtained for the naive and pre-exposed rats during the days when they were not disturbed, the data for both groups were combined to create one temperature profile for the no rectal probe data (n = 16). The original one-minute abdominal temperature recordings were averaged over two hours and the two-hourly means have been plotted. * Rectal probe pre-exposed + Brewer's yeast significantly different to Rectal probe pre-exposed + Saline (Tukey's, $P < 0.05$). # Rectal probe naive + Brewer's yeast significantly different to Rectal probe naive + Saline (Tukey's, $P < 0.01$).

Figure 2.2 shows the mean difference (bias) and limits of agreements of the rectal temperatures and abdominal temperatures measured at 18 hours after injection. The temperature values measured with the two methods differed in rats injected with Brewer's yeast (mean difference 0.04 ± 0.23 ; 95% limits of agreement -0.4 to 0.48) and in rats

injected with saline (mean difference -0.2 ± 0.25 ; 95% limits of agreement -0.68 to 0.29). Thus, the agreement interval between the two methods identified that the temperature values recorded in rats using a thermocouple probe inserted into the rectum could either be 0.7 °C greater or 0.5 °C lower than those obtained with a radiotransmitter implanted into the abdomen.

The average change in abdominal temperature, over the 5-hour period (10:00 – 15:00), of rats pre-exposed or naive to rectal temperature measurements and injected s.c. with Brewer's yeast was not significantly different from the change in abdominal temperature of rats not exposed to rectal temperature measurements and injected with Brewer's yeast (main effects of time ($F_{(59, 1062)} = 4.04$, $P < 0.0001$), treatment ($F_{(2, 18)} = 2.63$, $P = 0.10$) and their interaction ($F_{(118, 1062)} = 1$, $P = 0.49$)) (Figure 2.3A).

Rectal temperature measurement induced a significant increase in the abdominal temperature of rats injected s.c. with saline compared to when they did not receive rectal temperature measurements, i.e. when they were left undisturbed in their home cages (Figure 2.3B) (main effects of time ($F_{(59, 1711)} = 20.49$, $P < 0.0001$), treatment ($F_{(2, 29)} = 20.42$, $P < 0.0001$) and their interaction ($F_{(118, 1711)} = 7.02$, $P < 0.0001$)). After each rectal temperature measurement, the increase in abdominal temperature, of rats injected with saline that were pre-exposed or naive to rectal temperature measurements, occurred after a latent period of 5 - 10 minutes, peaked at 15 - 20 minutes with a mean maximum change of 0.66 ± 0.27 °C, and resolved after 35 - 50 minutes. The regression slopes plotted for the maximum change in abdominal temperature, recorded after each rectal temperature measurement against number of rectal temperature measurements significantly deviated from zero in only three of the sixteen rats. Thus, for most rats, the maximum change in abdominal temperature induced by rectal temperature measurement did not decrease with repeated rectal temperature measurements taken at hourly intervals.

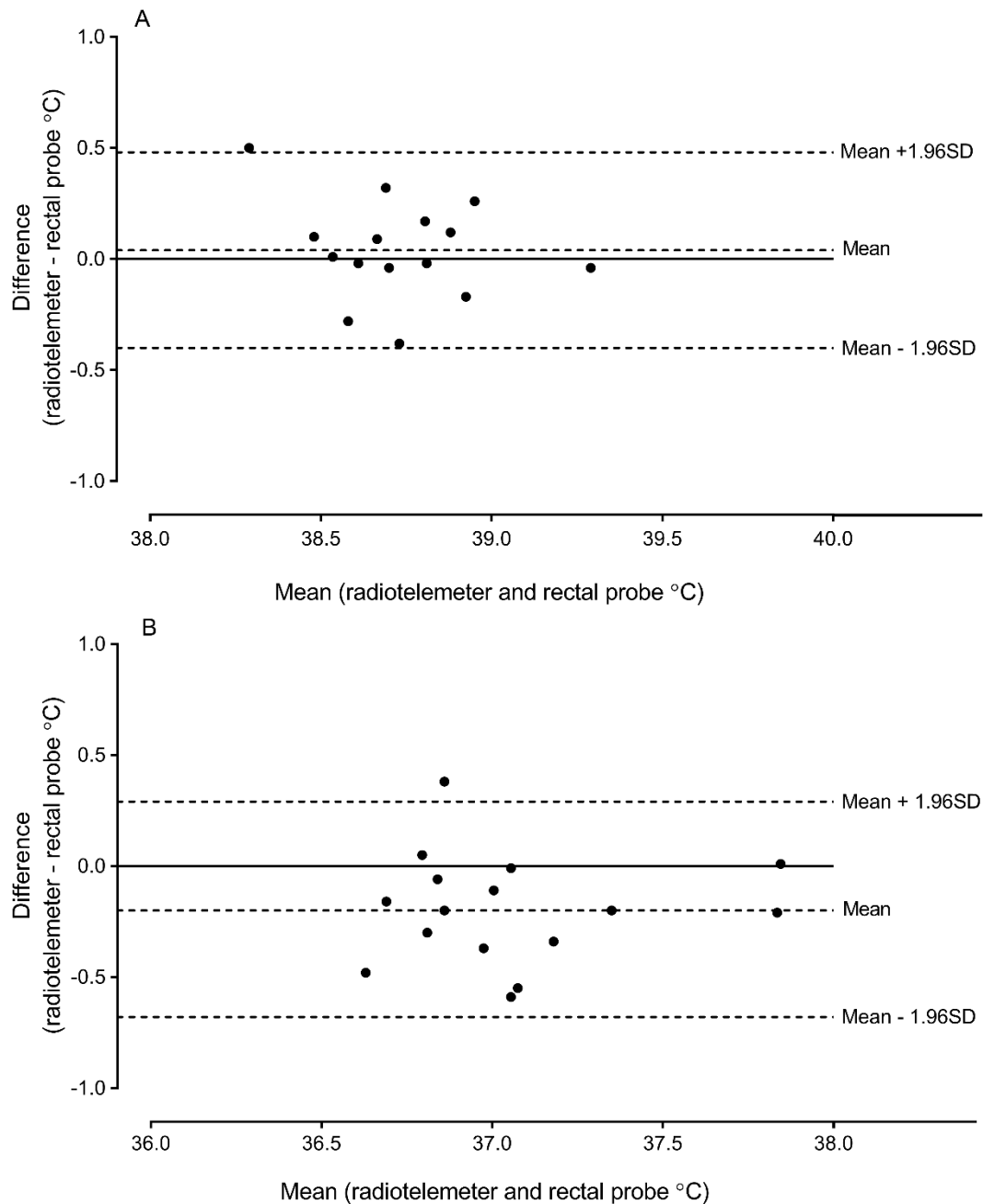


Figure 2.2: The mean difference (bias) and limits of agreements of the rectal temperatures and abdominal temperatures measured at 18 hours in (A) rats with a fever induced by Brewer's yeast (n=15) or (B) rats injected with saline (n=16). Rats were housed at an ambient temperature of 22 ± 2 °C. Black dots represent individual animals. Upper limit of agreement is calculated as the mean difference between two methods plus 1.96 (SD) of the difference. Lower limit of agreement is calculated as the mean difference between two methods minus 1.96 (SD) of the difference.

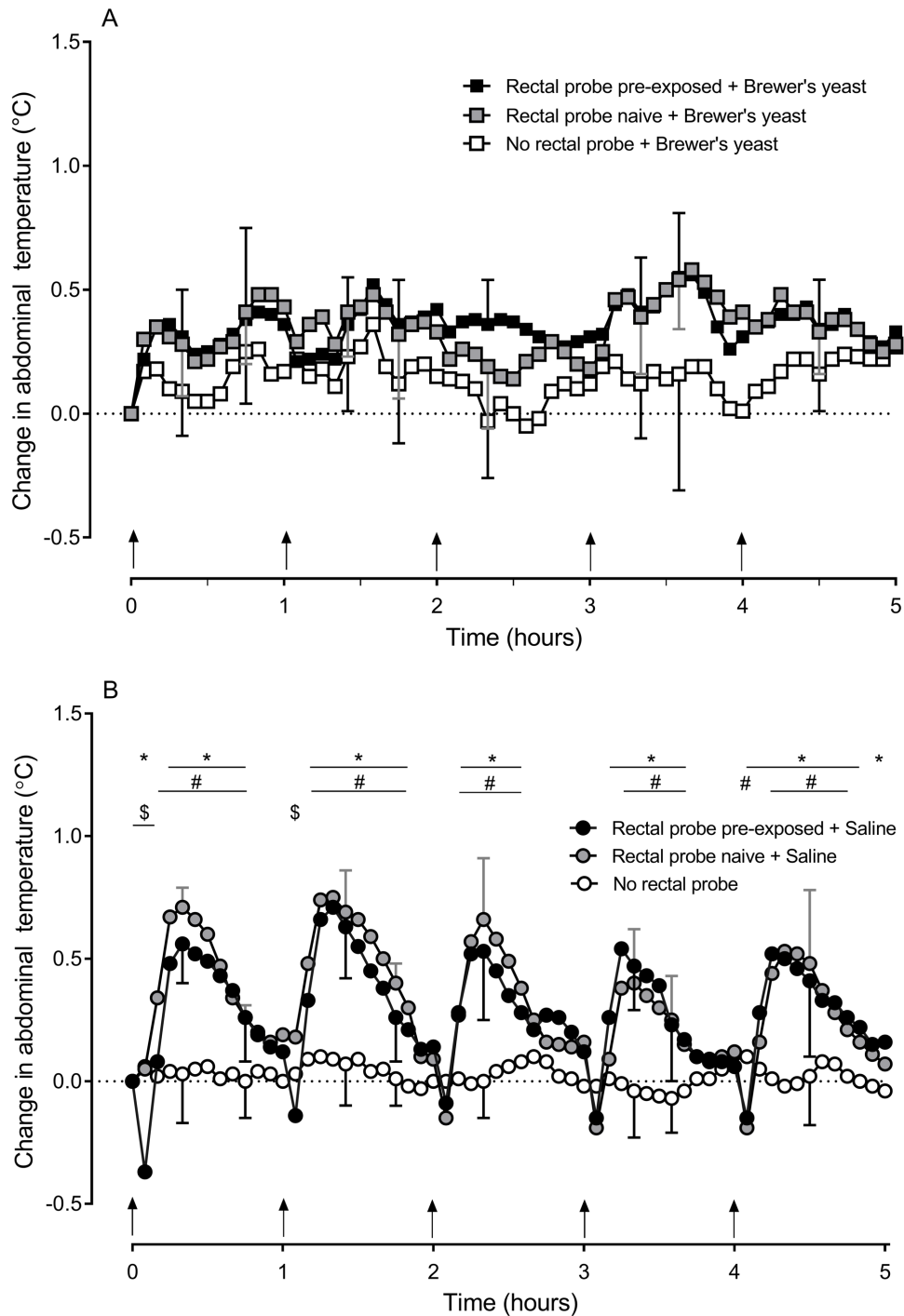


Figure 2.3: Change in abdominal temperature (mean and SD), shown in five-min intervals over five hours following rectal temperature measurement in (A) febrile rats injected s.c. with 4g/kg Brewer's yeast in saline ($n = 7$ pre-exposed; $n = 8$ naive) and (B) normothermic rats injected s.c. with saline ($n = 8$ pre-exposed; $n = 8$ naive). Pre-exposed rats had rectal temperatures taken once a day on the two days before the experiment. Naive rats received their first rectal temperature measurement only at the beginning of the experiment (at time T_0). $T_0 = 10:00$ clock time and $T_5 = 15:00$ clock time. The black arrows indicate the time of handling and rectal temperature measurements. Brewer's yeast and no rectal probe represent rats that received an injection but no rectal temperature

measurements (n = 6). No rectal probe (Figure 2.3B) represents the change in abdominal temperature when the rats that received saline were left undisturbed in their home cages; i.e. before exposure to rectal temperature measurements started. As there were no significant differences in the temperature profiles obtained for the naive and pre-exposed rats during the days when they were not disturbed, the data for both groups were combined to create one temperature profile for the no rectal probe data (n = 16). * Rectal probe pre-exposed + Saline significantly different to no rectal probe (Tukey's, $P < 0.05$). # Rectal probe naive + Saline significantly different to no rectal probe (Tukey's, $P < 0.05$). \$ Rectal probe pre-exposed + saline significantly different to Rectal probe naive + saline (Tukey's, $P < 0.05$). No significant differences were observed between the febrile groups.

2.4 Discussion

This study has shown that the procedure of measuring rectal temperature is associated with an increase in abdominal temperature (0.66 ± 0.27 °C) i.e. stress-induced hyperthermia, in normothermic rats, and that it is muted in rats during the plateau phase of fever (Figure 2.3). In normothermic rats, the stress-induced hyperthermia had a rapid onset (within 5 - 10 minutes), peaked at 15 - 20 min and lasted for 35 - 50 minutes after each rectal temperature measurement (Figure 2.3B). The exposure of rats to the procedure of rectal temperature measurement on two occasions before the start of the experiment did not significantly reduce the magnitude or duration of the stress-induced hyperthermia (Figure 2.3B). Similarly, five repeated measurements of rectal temperatures undertaken on the day of the experiment had no significant effects on the magnitude and duration of the stress-induced hyperthermia. The difference in temperature between rats injected with saline vs rats injected with Brewer's yeast, i.e. the amplitude of the fever, was smaller when temperature was measured using a thermocouple probe inserted into the rectum, than when temperature was measured using intra-abdominally implanted radiotelemeters. There was a significant positive linear association between temperatures recorded using a thermocouple probe inserted into the rectum and temperatures recorded using intra-abdominally implanted radiotelemeters in normothermic and febrile rats. Overall, the agreement interval between the two methods identified that the temperature values recorded in rats using a thermocouple probe inserted into the rectum could either be 0.7 °C greater or 0.5 °C lower than those obtained with a radiotransmitter implanted into the abdomen (Figure 2.2). Thus, the two methods of temperature measurement may yield qualitatively similar results but quantitatively different results.

The results obtained in this study showed that there is a clear and reproducible increase in body temperature (stress-induced hyperthermia) that developed after rectal temperature measurement in normothermic rats. Stress-induced hyperthermia is a well-established physiological response of various species of animals to different stressful events such as: restraint, frustration (Shibata and Nagasaka, 1982, Hashimoto et al., 2001, Parrott and Lloyd, 1995), cage-switching (Long et al., 1990c, Morimoto et al., 1991), exposure to an open field (Butterweck et al., 2003, Grundmann et al., 2006, Kluger et al., 1987, Rowsey et al., 2001), tail-suspension (Liu et al., 2003), capture (Meyer et al., 2008), and rectal temperature measurement (Bae et al., 2007, Dallmann et al., 2006, Poole and Stephenson, 1977, Hale et al., 2003). While hyperthermia appears to be a common physiological response in all of these stress paradigms, the magnitude of the stress-related increase in body temperature appears to be dependent on the intensity, modality and duration of the stressful stimulus and may vary across species [for review see Bouwknecht et al. (2007), Vinkers et al. (2008)]. The magnitude and duration of the hyperthermia noted in normothermic rats in the present study (between 0.5-1.5 °C) is consistent with those reported in other studies that used rectal probes for measuring temperature (Bae et al., 2007, Poole and Stephenson, 1977).

The findings from this study have important implications for the use of thermocouple probes to measure rectal temperature in pharmacological antipyretic screening studies. In these studies, rats are usually injected with either Brewer's yeast or saline and 18 hours after they are injected with the antipyretic agent being tested (Hassan et al., 2015a, Keri et al., 2010, Pingsusaen et al., 2015). The efficacy of the antipyretic agent is determined by comparing the rectal temperatures of the treated febrile rats and their normothermic controls. Similar body temperature measurements in the treated febrile rats and normothermic controls would indicate that the antipyretic agent being tested is indeed effective. Results from this study show that rectal temperature measured using a thermocouple probe underestimated the amplitude of fever and that normothermic rats injected with saline have a significant increase in abdominal temperature for 5 - 50 minutes after each rectal temperature measurement; maximum change of 0.66 ± 0.27 °C occurred 15 - 20 min after the measurement (Figure 2.3B). Thus, measurement of rectal temperatures, which report a smaller fever amplitude, coupled with a stress-induced increase in body temperature in control rats may potentially lead to investigators erroneously reporting a positive effect of the antipyretic agent under investigation,

especially if the rectal temperature measurements are taken at intervals of less than 60 min as has been reported in some studies [for example see (Josa et al., 2001, Liu et al., 2016, Pingsusaen et al., 2015, Zakaria et al., 2006)]. The majority of the pharmacological antipyretic screening studies however record rectal temperatures in 60 min intervals. As shown in our study, the hyperthermic response resolves between 40 - 50 min after rectal temperature measurement, hence values measured at hourly intervals are less likely to reflect the stress-induced hyperthermia, induced by the rectal temperature measurement. A study investigating the neuroendocrinological responses of hyperthermia in stressed mice also showed that corticosterone levels returned to pre-stress levels 60 min after stress induction (Veening et al., 2004). However, stress induced by rectal temperature measurement is also associated with a concomitant increase in concentrations of the pro-inflammatory cytokines IL-1 β and IL-6. Therefore, it is important to note that, even if the stress-induced hyperthermia resolves within 60 min, the basic biochemical make-up and physiological responses of a stressed rat will be different from those of an undisturbed rat. These changes could impact on results, particularly in studies investigating the pathophysiological mechanisms mediating fever or the potential effects of various antipyretic agents.

Subjecting the rats to rectal temperature measurements on two previous occasions in an attempt to alleviate stress-induced hyperthermia, as is commonly done in other studies (Souza et al., 2002, Tomazetti et al., 2005), did not change the magnitude or duration of the hyperthermia induced by the rectal temperature measurements. Similarly, repeating the procedure five times, at 60 min intervals, on the day of the experiment did not significantly alter the magnitude or duration of the hyperthermia noted following each rectal temperature measurement. Although an effective duration of habituation to the procedure of rectal temperature measurement was not strictly investigated in this study, two days before the experiment may be too short a period to pre-expose the rats and habituate them to the procedure. Others have similarly reported no reduction in the hyperthermic response of rats pre-exposed to rectal temperature measurement 15 times over a period of five days (Bae et al., 2007).

While the hyperthermic response to rectal temperature measurement is prominent, long-lasting and reproducible in normothermic rats (Figure 2.3B) it appears muted in rats during the plateau phase of fever induced by s.c. administration of Brewer's yeast (Figure

2.3A). Results obtained from studies using other stressful events, such as cage-switching and exposure to an open field, have reported conflicting findings regarding the possible involvement of prostaglandins, the terminal mediators of fever, in mediating stress-induced hyperthermia (Kluger et al., 1987, Singer et al., 1986, Morimoto et al., 1991, Lecci et al., 1990, Parrott and Lloyd, 1995, Vinkers et al., 2009). If prostaglandins are indeed involved in mediating stress-induced hyperthermia then the absence of hyperthermia following rectal temperature measurement in febrile rats might be related to saturation of prostaglandin receptors. Alternatively, it has also been previously proposed that the absence of stress-induced hyperthermia in febrile rats may be related to a ceiling effect i.e. a maximally reachable temperature above which no further rise is possible (Vinkers et al., 2009). However, because rats injected with Brewer's yeast in this study had earlier attained higher temperatures i.e. at the peak of the fever and additionally showed an ability to raise their temperature during the plateau phase (Figure 2.1 and 2.3B), it is unlikely that a ceiling effect for body temperature contributed to the absence of the hyperthermia observed in our study.

The proposed sources of heat production associated with stress-induced hyperthermia in rats include: (1) increased non-shivering thermogenesis in brown adipose tissue (BAT), (2) cutaneous vasoconstriction to reduce heat loss (mediated by sympathetic outflow especially to the tail), and (3) activation of the hypothalamic-pituitary-adrenal (HPA) axis to increase production of thermogenic hormones (Bi, 2014, Morrison, 2016b, Morrison, 2016a, Oka et al., 2001). In this study, rats were housed in a sub-thermoneutral environment (22 ± 2 °C). Rats housed in sub-thermoneutral environments use sympathetic activation of cutaneous vasoconstriction and BAT thermogenesis activated through the preoptic area, DMH and the medullary raphe to maintain normal core body temperatures (Ootsuka and McAllen, 2006, Nakamura and Morrison, 2008, Rathner et al., 2001, Morrison, 2016b, Morrison, 2016a). The same heat generating mechanisms and brain regions are also employed in maintaining the febrile state during infection-induced fever.

The “law of initial values” postulated by Wilder in 1931 (Wilder, 1931), states that the direction and magnitude of a physiological response depends, to a great extent, on the initial (pre-stimulus) values. Thus, the existence of elevated initial (pre-stimulus) values may constrain further increases in a physiological response and therefore lessen the

magnitude of the response observed when a stimulus is applied. As illustrated in Figure 2.4, stress-induced hyperthermia, exposure to cold and infection-induced fever share common descending pathways and effectors for thermogenesis (DiMicco et al., 2006, Zaretskaia et al., 2002, Zaretskaia et al., 2003, Cerri and Morrison, 2006, Kataoka et al., 2014, Nakamura, 2011, Nakamura et al., 2005a, Sarkar et al., 2007). Applying the law of initial values to the findings of the present study, the absence of the stress-induced hyperthermia in the febrile rats may be related to a pre-existent background activation of heat generating mechanisms for cold defence and maintenance of fever.

In the comparison of rectal thermometry and biotelemetry, the agreement interval between the two methods showed that rectal temperature recorded using a thermocouple probe could either be as much as 0.7 °C greater or 0.5 °C lower than temperatures obtained with a radiotransmitter implanted into the abdomen (Figure 2.2). Others have reported mean differences in excess of 1 °C in normothermic rats (Dilsaver et al., 1992). The accuracy of temperatures recorded using a thermocouple probe inserted into the rectum is heavily dependent on the probe insertion depth. Temperatures measured closer to the anal opening (further away for the liver) are lower and a less accurate representation of core body temperature (Dilsaver et al., 1992). Faecal material, involuntary/reflexive smooth muscle movements in the colon and at times slight movements of the rat itself may change the depth of the probe during measurement. Thus, the accuracy of the rectal temperature measurements recorded using a thermocouple probe is contingent on the technique and consistency of the researcher. These factors are however difficult to standardize because not all animals cooperate or respond in the same way every time. In this regard, although surgically implanted radiotelemeters are more expensive than thermocouple probes, they appear to be a more reliable method of core body temperature measurement in rodents, particularly in studies measuring temperature responses over a prolonged period of time (Clement et al., 1989).

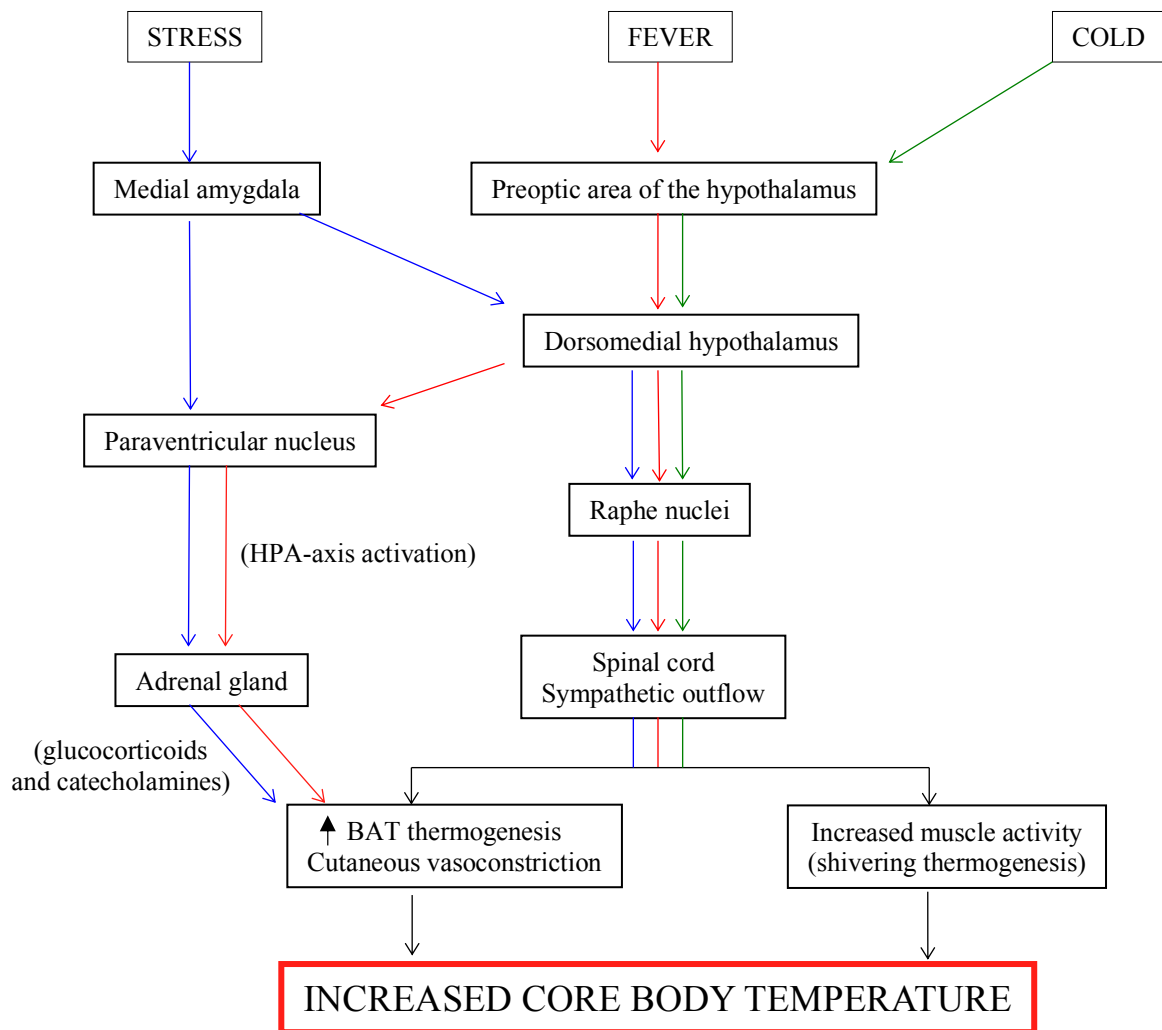


Figure 2.4: Neural pathways mediating thermogenesis induced by stress, fever and cold exposure. Stress activates the medial amygdala, which in turn activates the dorsomedial hypothalamus (DMH) and paraventricular nucleus (PVN). Fever and cold exposure both activate the preoptic area (POA) of the hypothalamus, which in turn activates the DMH (and partly raphe nuclei, not shown). The DMH activates the raphe nuclei in the medulla oblongata to stimulate sympathetic premotor neurons in the spinal cord and increase brown adipose tissue (BAT) metabolism, cutaneous vasoconstriction and shivering. The DMH also activates the hypothalamic-pituitary adrenal (HPA) axis and stimulates the release of thermogenic hormones such as the glucocorticoids (e.g. corticosterone) and the catecholamines (e.g. adrenaline). Hormones of the adrenal gland then activate BAT thermogenesis, metabolism and cutaneous vasoconstriction leading to an increase in core body temperature.

In conclusion, the findings from this study show that the procedure of rectal temperature measurement induces a clear hyperthermic response in normothermic rats, but not in rats during the plateau phase of fever. These findings are important particularly for antipyretic screening studies where conclusions are drawn based on comparisons of temperature responses of febrile rats and their normothermic controls. The use of rectal thermometry in these studies may affect the reliability of results especially if short-spaced, intermittent rectal temperature measurements are used.

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CHAPTER THREE

Pyrogenic models used during antipyretic testing: A characterisation of the fever, sickness behaviour and inflammatory mediators associated with Brewer's yeast administration

Data and ideas from this chapter have been published as a scientific paper: Dangarembizi, R., Erlwanger, K H., Rummel, C., Roth, J., Madziva, M T and Harden, L M. Brewer's yeast: a potent inducer of fever and sickness behaviour through activation of inflammatory mediators in the brain. *Brain Behaviour and Immunity* **68**; 211-223.

3.1 Introduction

For almost a century, *Saccharomyces cerevisiae* (*S. cerevisiae*) (commonly known as Brewer's or Baker's yeast) has remained the standard pyrogen for inducing pyrexia in pharmacological, especially ethnopharmacological, studies screening antipyretics in rats (Nicolle, 1925, Tomazetti et al., 2005, Loux et al., 1972, Aouey et al., 2016, Abotsi et al., 2016, Liu et al., 2016). Pharmacological antipyretic testing guidelines in rats recommend that the test drug or the standard drug be administered 18 hours after subcutaneous injection of yeast and rectal temperatures be recorded at time intervals of 30 – 60 min, for five hours thereafter (Thangaraj, 2016, Vogel, 2002). Although Brewer's yeast is used extensively in antipyretic testing protocols in rats, the sickness response to this pyrogen has not been fully characterised. Results from **Chapter 2** support the reports in current literature [see section 1.6.2.1 for review of literature] which show that Brewer's yeast induces fever, but there is very little information available describing the characteristics of the fever and sickness behaviour across the range of Brewer's yeast doses that are commonly used.

In addition to the fever and sickness behaviour not being fully characterised, the spatiotemporal inflammatory profile associated with yeast administration has also not been fully described. Thus, it is unclear which inflammatory mediators are present around the recommended antipyretic administration time point of 18 hours after administration of Brewer's yeast. As outlined in Chapter 1 (section 1.5), reported mechanisms of action of antipyretics include: (1) inhibiting the enzyme cyclooxygenase (COX) and reducing the levels of PGE₂ within the hypothalamus, (2) reducing pro-inflammatory mediators, and (3) enhancing anti-inflammatory signals (Aronoff and Neilson, 2001). The activation of transcription factors controls the expression of immune response genes that ultimately leads to the synthesis and release of the inflammatory mediators regulating fever [for review see Rummel (2016)]. The most important inflammatory transcription factors implicated in mediating the inflammatory response associated with fever include: nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT)3 and NF-IL6 [for review see Rummel (2016)]. Thus, collectively characterizing the fever-curve and the spatiotemporal profile of inflammatory mediators induced following yeast administration would provide valuable insight for studies using Brewer's yeast to screen antipyretics and determine their mechanism of action.

Thus, I have chosen to characterise the sickness response across a range of Brewer's yeast doses and to determine the spatiotemporal activation of inflammatory mediators in the periphery and key brain regions implicated in mediating fever, namely the hypothalamus and OVLT. Results obtained in my study show that Brewer's yeast administration has the capacity to induce fever, lethargy, anorexia and growth-stunting lasting at least six days. Eighteen hours after injection corresponded to the plateau phase of the fever where the core body temperature of rats receiving the high and medium doses of Brewer's yeast was consistently greater than the core body temperature of rats that received saline. The time-course of the inflammatory changes showed a marked increase in the synthesis of pro-inflammatory cytokines, key prostaglandin-synthesizing enzymes and transcription factors in the periphery and brain between 2 and 18 hours after Brewer's yeast administration.

3.2 Methods

3.2.1 Animals

Male Sprague Dawley rats were purchased from the National Health Laboratory Service (Johannesburg, South Africa) and housed in the Central Animal Service Unit of the University of the Witwatersrand, Johannesburg. The rats were housed individually in acrylic cages lined with wood shavings in a temperature-controlled room (22 ± 2 °C) on a 12h:12h light/dark cycle (lights on at 06:00 clock time). The rats had access to rodent pellets (Epol, Johannesburg, South Africa) and water *ad libitum*. Rats were allowed to acclimate to their environment for seven days before surgery. On the day of injection of the pyrogen, rats had a body mass of between 250 and 300 g. All experiments were performed in accordance with the principles of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Screening Committee (ethics clearance number 2014/10/C; see Appendix A).

3.2.2 Pyrogen administration

Rats received a single subcutaneous (s.c.) injection of Brewer's yeast (Sigma Aldrich, St Louis, USA: Lot number SLBC 5519V) reconstituted in a vehicle of 0.9% sterile, non-pyrogenic saline (Sabax, Johannesburg, South Africa) to a final concentration of 20% (w/v). The yeast

solution was injected in the dorsum of the neck at a volume of 20 ml/kg (4 g yeast/kg body weight), 10 ml/kg (2 g yeast /kg body weight) or 2 ml/kg (0.4 g yeast /kg body weight). The high (4 g/kg) and intermediate (2 g/kg) doses of Brewer's yeast are commonly used for inducing fever in young rats (~200-250 g) in pharmacological studies (Keri et al., 2010).

3.2.3 *Body temperature and cage activity*

As previously described by Hopwood et al. (2009), core body temperature and cage activity of rats were recorded using sterile, wax-coated temperature-sensitive radiotelemeters (TA10TA-F40, Data Sciences, St Paul, MN, USA). The radiotelemeters had been implanted intra-abdominally, with rats under general anaesthesia induced by an intramuscular (i.m.) injection of ketamine hydrochloride (80 mg/kg, Anaket-V, Bayer, South Africa) and xylazine (20 mg/kg, Chanazine, Bayer, South Africa). After surgery, the rats received a single i.m. injection of an analgesic (Temgesic®; Schering-Plough, Johannesburg, South Africa), and were given a week to recover from the surgery. The radiotelemeters were calibrated in a water bath at 35 °C and 39 °C by the manufacturer (Data Sciences, St Paul, MN, USA) to an accuracy of 0.1 °C.

3.2.4 *Body mass and food intake*

Body mass and food intake were measured daily, using an electronic scale (Diamond, US) accurate to 1 g, two hours before the beginning of the dark phase of the circadian cycle. To measure food intake, 200 g of standard rat chow were given to each rat and the daily intake was the difference between the initial weight of food at the start (200 g) and the food remaining after 24 hours.

3.2.5 *IL-6 and TNF- α bioassays*

To determine the level of TNF- α in plasma, a bioassay based on the dose-dependent cytotoxic effect of TNF on a mouse fibrosarcoma cell line WEHI 164 subclone 13 (Espevik and Nissen-Meyer, 1986) was performed. Serial dilutions of plasma samples or a murine TNF- α standard were incubated for 24 hours in 96 microtitre wells seeded with 50 000 actinomycin D-treated WEHI cells. Cell viability after 24 hours was measured using the dimethyl tetrazolium bromide

(MTT) assay [as previously described by (Holt et al., 1991)]. The detection limit of the assay, after factoring in the dilution of samples, was 6 pg/ml.

IL-6 bioactivity was determined using a bioassay that is based on the dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line (Aarden et al., 1987). Serial dilutions of plasma samples or different concentrations of the human IL-6 standard (89/548; National Institute of Biological Standards and Control, UK) were incubated for 72 hours in wells that had been seeded with 5000 B9 cells. According to the National Institute of Biological Standards and Control, one microgram of the IL-6 standard is equivalent to 100 000 international units (IU). Viable cells in each cell were measured using the MTT assay. The detection limit of the assay, after factoring in the dilution of samples, was 3 IU for IL-6/ml.

The levels of TNF- α and IL-6 determined in this study are not a reflection of the absolute concentrations of the cytokines but rather their bioactivity according to established international standards. The specificity of the assays was confirmed by neutralizing some stimulated microcultures with one microgram of TNF binding protein or 10 μ L of antiserum against rat-IL-6 (Ross et al., 2003, Harden et al., 2011).

3.2.6 Tissue preparation

Coronal brain sections (20 μ m) were cut at -20 °C on a cryostat (2800 Frigocut-E, Cambridge Instruments, West Germany) and thaw mounted onto poly-L-lysine-coated glass slides. Brain sections used in this study were prepared from areas encompassing the vascular organ of the lamina terminalis (OVLT) according to the stereotactic atlas of the rat brain (Paxinos and Watson, 1998). Twelve sections were collected per rat. The same brains used for immunohistochemistry were also used for RT-PCR and to do so, 35 consecutive, frozen, 30 μ m sections of the hypothalamus were thaw-mounted on glass slides and the hypothalamus was carefully dissected out and used for RNA extraction. Sections were stored at – 55 °C until processing.

3.2.7 Immunohistochemistry

3.2.7.1 General procedure

Frozen brain sections were air dried for 10 min and fixed in 2% paraformaldehyde (PFA; Merck, Darmstadt, Germany) followed by three 5-min washes with phosphate-buffered saline (PBS). The sections were then blocked with 10% Normal Donkey Serum (NDS; Biozol, Germany) and 0.3% Triton X-100 (0.1% for NF-IL6 and NF- κ B, and 0.3% for COX-2 and STAT3). Following this, sections were incubated with primary antibody solutions overnight at 4 °C (details of antibodies are as described in Table 3.1). The specificity of the respective antibodies has previously been tested (Damm et al., 2011, Fuchs et al., 2013, Rummel et al., 2006, Rummel et al., 2011). After three successive 5-min washes with PBS, sections were incubated with secondary antibodies conjugated with fluorescent dyes (Cy3 and Alexa-488) for 2 hours at room temperature. This was followed by nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) stain (1:8000) to clearly visualize the nucleus and to demonstrate the nuclear localization of immunoreactive protein (Mabtec GmbH, Göttingen, Germany) and three final 5-min washes with PBS after which sections were cover-slipped with glass coverslips and glycerol/PBS solution (Citifluor, Ltd, London, UK). Sections were stored at 4 °C pending microscopic analysis.

3.2.7.2 Colocalization

Double immunohistochemistry was performed to determine the contribution of endothelial cells to the NF-IL6, STAT3 and COX-2 immunoreactive cell population. The antibodies for NF-IL6, STAT3 or COX-2 were mixed with the cell marker antibody (dilutions given in Table 3.1) before the first incubation (20 – 22 hours). NF-IL6 and STAT3 was visualized with Cy3-conjugated anti-rabbit IgG, COX-2 was visualized with Cy3-conjugated anti-goat IgG, endothelial cell markers with Alexa-488-conjugated anti-rabbit or anti-sheep IgG as secondary antibodies (1:500 dilution each). In control experiments, the sections were incubated with the primary antibody mixture in which one of the primary antibodies was substituted with non-immunized animal IgG (goat IgG for COX-2, rabbit IgG for NF-IL6). Inappropriate cross-reaction between antibodies was confirmed to be negligible.

Table 3.1: Antibodies used for immunohistochemistry

Antibody	Dilution	Catalog number
<i>Primary antibodies</i>		
NF-IL6 rabbit polyclonal IgG	1: 6000	sc-150-G Santa Cruz Biotechnology, Santa Cruz, CA
vWF rabbit polyclonal IgG	1:5000	008202 Dako Deutschland, GmbH, Hamburg, Germany
vWF sheep polyclonal IgG	1:2000	SARTW-IG Affinity Biologicals, Ancaster, Ontario, Canada
COX-2 goat polyclonal IgG	1:2000	sc-1747 Santa Cruz Biotechnology, Santa Cruz, CA
STAT-3-rabbit polyclonal IgG	1:3000	sc-482 Santa Cruz Biotechnology, Santa Cruz, CA
NF-κB-rabbit polyclonal IgG	1:500	sc-109 Santa Cruz Biotechnology, Santa Cruz, CA
<i>Secondary antibodies</i>		
Cy3-conjugated anti-rabbit IgG	1:600	711-165-152 Jackson Immuno Research West Grove, PA
Cy3-conjugated anti-goat IgG	1:600	705-165-147 Jackson Immuno Research West Grove, PA
Alexa 488-conjugated anti-rabbit IgG	1:500	AZA11055 MobiTec GmbH, Göttingen, Germany
Alexa 488-conjugated anti-sheep IgG	1:500	AZA11015 MobiTec GmbH, Göttingen, Germany

vWF = von Willebrand factor, NF-IL6 = nuclear factor for IL-6, NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells, IgG = immunoglobulin G, STAT-3 = signal transducer and activator of transcription-3

3.2.8 Microscopic analysis

Processed sections were analysed using a light/fluorescent microscope (Olympus BX50, Olympus Optical, Hamburg, Germany). The microscope was equipped with filter sets to allow detection of the fluorescent conjugates used (Cy3, Alexa 488 and DAPI). All images were taken using a black and white Spot Insight camera (Diagnostic Instruments, Visitron Systems, Puchheim, Germany) using the Metamorph 5.05 software. Metamorph 5.05 was also used to combine individual images into the red/green/blue (RGB) colour figure plates (staining was performed for all animals at the same time and for quantification purposes all images were processed the same way using Adobe Photoshop CC 2017 (Adobe Systems Incorporated, San Jose, CA, USA)).

3.2.9 Real-time PCR

RNA extraction from hypothalamic tissue was done using TRIzol (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA (1 µg) was transcribed in a 10 µL reaction mix comprising of 50U murine leukemia virus (MLV) reverse transcriptase, 50 mM random hexamers and 10 mM dNTP mix (Applied Biosystems, Foster City, CA, USA). Qualitative PCR was thereafter performed in duplicate using a preoptimised primer/probe mixture (Taqman Gene Expression Assay; Applied Biosystems, Foster City, CA, USA) and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using a StepOne RT-PCR System (Applied Biosystems Foster City, CA, USA) (Damm et al., 2011). β -actin was used as the housekeeping gene for normalizing cDNA concentrations from the different sample reactions. Sample values were derived from comparison with a reference sample (from the same experiment and designated the value of 1). Samples value represented an x-fold difference from this reference (Damm et al., 2011). The assay IDs for the analysed genes are as follows: NF-IL6 (Rn00824635_s1), I κ B α (Rn01473658_g1), SOCS3 (Rn00585674_s1), COX-2 (Rn00568225_m1), mPGES (Rn00572047_m1), TNF- α (Rn99999017_m1), IL-1 β (Rn00580432_m1), and IL-6 (Rn01410330_m1); (Applied Biosystems, Foster City, CA, USA).

3.2.10 Experimental procedures

3.2.10.1 Experiment 1: Characterisation of fever and sickness behaviour

Rats were assigned randomly to receive one of three different doses of 20% (w/v) Brewer's yeast solution (20 ml/kg = 4 g/kg (n = 9), 10 ml/kg = 2 g/kg (n = 5) or 2 ml/kg = 0.4 g/kg (n = 6)) or saline (20 ml/kg of 0.9% (n = 6). Injections were administered at 16:00, two hours before lights off at 18:00. Core body temperatures, cage activity, food intake and body mass were monitored for 3 days before and 6 days after injection.

3.2.10.2 Experiment 2: Spatiotemporal characterisation of inflammatory mediators

Separate groups of rats were assigned randomly to receive one of two doses of Brewer's yeast solution (4 g/kg or 2 g/kg) or saline, before brain tissue was collected under terminal

anaesthesia induced with an i.p. injection of 1 ml sodium pentobarbital (Euthanase, 200 mg/ml; Kyron Laboratories (Pty) Ltd., South Africa). Blood and brain samples were collected at: 2, 8, 18 and 72 hours after injection; with five to seven rats euthanased per time point for each of the intervention groups. These four time points were chosen to represent four different phases of the febrile response the early phase (2 hours), the peak (8 hours), the plateau phase (18 hours) and defervescence (72 hours). Blood was collected by cardiac puncture into EDTA tubes after which the rats were transcardially perfused using ice cold saline (0.9%) for 2 min. Whole brains were recovered and quickly frozen on powdered dry-ice and stored at -80 °C (Damm et al., 2011).

3.2.11 Data analysis

All data are expressed as mean and SD and were analysed using GraphPad Prism (version 7.0, GraphPad Software Inc, USA). Significance was set at $P < 0.05$. For statistical purposes, the original 5-min temperature recordings were averaged over successive two-hour periods. Abdominal temperature responses were plotted as abdominal temperature-time curves in one-hour intervals. The 5-min nocturnal activity counts for each rat were summed for 12 hours (18:00 – 06:00) and the summed values were averaged for three nights before the day of injection, when the rats were undisturbed in their home cages. Change in nocturnal cage activity was then calculated as the difference between the night-time activity following injection and the average night-time activity in the three nights before injection, and expressed as a percentage of the average pre-injection activity.

Food intake was calculated as grams of food consumed in 24 hours per 100 g of body mass and expressed as a percentage change from the mean daily food intake measured over three days before injection. Twenty-four-hour change in body mass (g) was determined by subtracting the body mass measured at 17:00 on each day after the injection from the body mass measured immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass.

The two-hourly temperature means and changes in nocturnal cage activity, food intake and body mass were analysed by two-way repeated measures analysis of variance (ANOVA) with intervention and time as main effects. Tukey's multiple comparisons *post hoc* test was used to

detect differences when the ANOVA detected significant main effects or interactions. To determine whether the change in cage activity, food intake and body mass on each of the six days after injection was significantly different from pre-injection values, a one-sample t-test was performed for each of the experimental groups.

Cytokine levels and relative mRNA expression were compared by two-way ANOVA with treatment and time as main effects. Tukey's multiple comparisons *post hoc* test was used to detect differences when the ANOVA detected significant main effects or interactions. Where cytokine levels were undetectable, samples were assigned a value equivalent to the detection limit of the assay. Plasma cytokine data that were not normally distributed were log-transformed prior to statistical analysis. The data presented are non-transformed means and SD, but the associated significance values were derived from log-transformed data.

3.3 Results

3.3.1 *Experiment 1 - Brewer's yeast induces fever, lethargy, anorexia and body mass stunting*

3.3.1.1 *Body temperature and nocturnal activity*

Figure 3.1 shows the core body temperature over six days for rats that received s.c. injections of Brewer's yeast or saline. The main effects of time ($F_{(72, 1584)} = 121.10, P < 0.0001$), treatment ($F_{(3, 22)} = 54.02, P < 0.0001$) and interaction ($F_{(216, 1584)} = 13.83, P < 0.0001$) were significant such that rats injected with Brewer's yeast developed a fever while rats injected with saline did not. The core body temperature of rats injected with the high dose of Brewer's yeast (4 g/kg) initially decreased to 36.44 ± 0.60 °C. The decrease in core body temperature lasted for 3 hours and was followed by a fever, which peaked at 39.17 ± 0.23 °C ~ 8 hours after injection. Core body temperature of rats injected with the medium (2 g/kg) or low (0.4 g/kg) doses of Brewer's yeast started to increase after a latent period of ~ 3 hours and peaked at ~ 8 hours after injection at 39.16 ± 0.20 °C and 38.66 ± 0.30 °C respectively. The duration of the fever was dependent on the dose of yeast injected. Fever induced by the 0.4 g/kg dose of yeast persisted for the first night and day after injection ($P < 0.05$, Tukey), while fever induced by the medium dose (2 g/kg) and high dose (4 g/kg) persisted for five ($P < 0.05$, Tukey) and six ($P < 0.05$, Tukey) days respectively, including the first two nights.

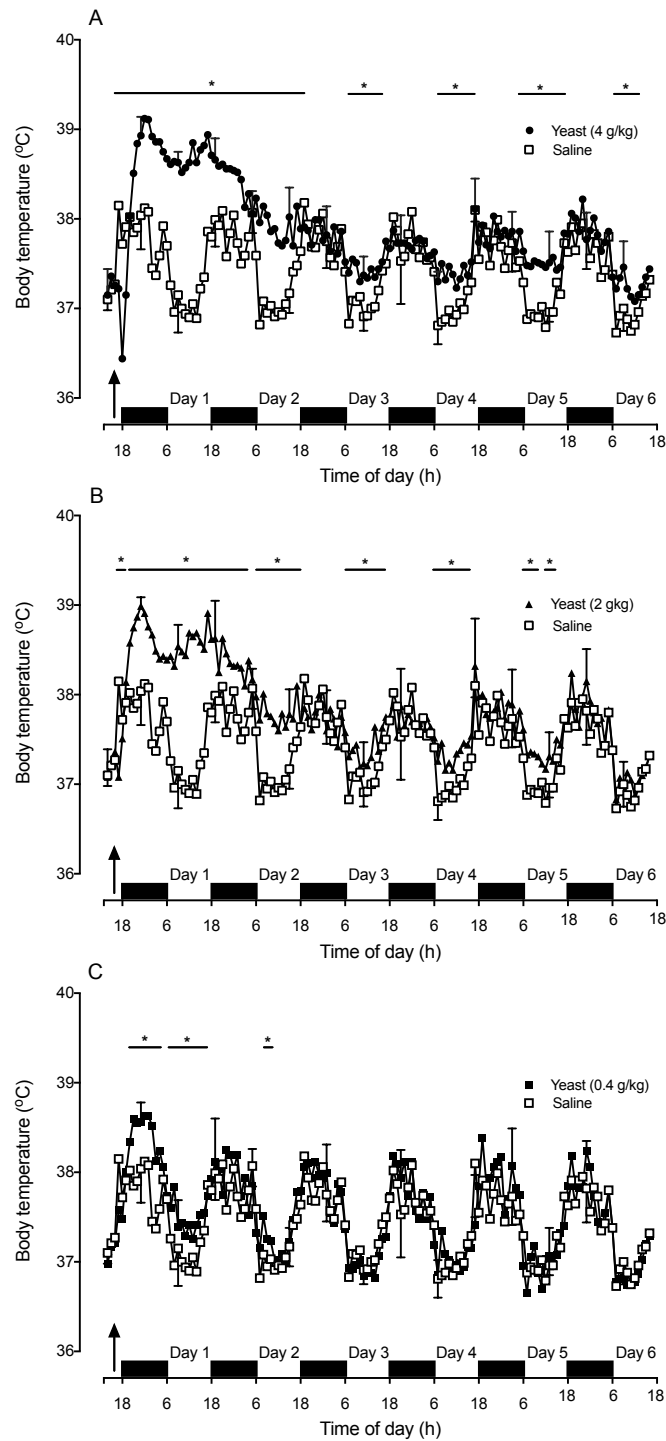


Figure 3.1: Core body temperature (mean and SD) of rats over six days after subcutaneous injection of (A) 4 g/kg Brewer's yeast (n = 9), (B) 2 g/kg Brewer's yeast (n = 5) and (C) 0.4 g/kg Brewer's yeast (n = 6) or saline (n = 6). Core body temperatures were plotted in one-hour intervals. The black arrow indicates injection time and black bars indicate lights off (18:00-06:00 clock time). * significantly different from saline ($P < 0.05$, Tukey).

Figure 3.2 shows that s.c. injection of Brewer's yeast and saline both induced a significant decrease in nocturnal cage activity, but the decrease following Brewer's yeast injection was significantly greater, on the first night after injection, than that following the saline injection, and lasted longer (main effects of time ($F_{(5, 110)} = 54.63$, $P < 0.0001$), treatment ($F_{(3, 22)} = 74.76$, $P < 0.0001$) and interaction ($F_{(15, 110)} = 7.53$, $P < 0.0001$). Rats receiving the high and medium dose of Brewer's yeast decreased nocturnal cage activity by $\sim 80\%$ on the first night after injection.

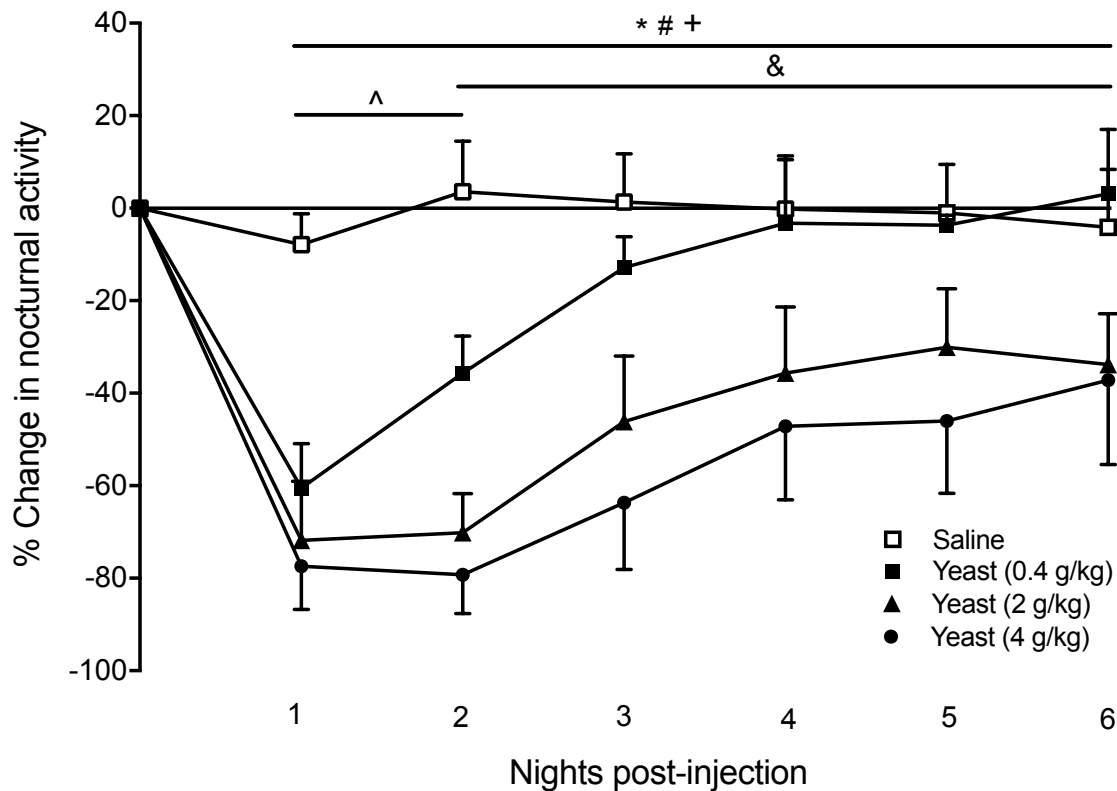


Figure 3.2: Change in nocturnal activity (mean and SD) of rats over six days after subcutaneous injection of either 4 g/kg Brewer's yeast ($n = 9$), 2 g/kg Brewer's yeast ($n = 5$), 0.4 g/kg Brewer's yeast ($n = 6$), or saline ($n = 6$). *Indicates differences between 4 g/kg Brewer's yeast and saline ($P < 0.0001$, Tukey); # indicates differences between 2 g/kg Brewer's yeast and saline ($P < 0.001$, Tukey); ^ indicates differences between 0.4 g/kg Brewer's yeast and saline ($P < 0.0001$, Tukey); + indicates differences between 4 g/kg and 0.4 g/kg Brewer's yeast ($P < 0.05$, Tukey); & indicates differences between 2 g/kg and 0.4 g/kg Brewer's yeast ($P < 0.01$, Tukey). One sample t test performed on each experimental group at each time point indicated that the decrease in cage activity: (i) never returned to pre-injection values for rats that received 4 g/kg or 2 g/kg Brewer's yeast; (ii) returned to pre-injection values on night 4 post injection for rats injected with 0.4 g/kg Brewer's yeast; and (iii) for rats injected with saline was only reduced compared to pre-injection values on the first night after injection.

From the third night onwards rats injected with the high or medium dose of Brewer's yeast gradually became more active, but activity never fully recovered to pre-injection levels, as by the end of the sixth night they remained less active than before the injections ($t = 6.1$, $P = 0.0003$ high dose; $t = 6.9$, $P = 0.002$ medium dose). Rats receiving a s.c. injection of the low dose of Brewer's yeast decreased nocturnal cage activity by approximately 60% on the first night after injection, which was significantly less than the decrease in nocturnal activity for rats that received the high dose of Brewer's yeast ($P < 0.05$, Tukey). Unlike that noted for the high and medium doses of Brewer's yeast, activity recovered, from its initial decrease, such that, from the fourth night after injection rats that received the low dose of Brewer's yeast had nocturnal activity levels similar to those before the injections ($t = 0.57$, $P = 0.59$).

3.3.1.2 Food intake and body mass

For the three days before receiving an injection the average daily food consumption of the rats per 100 g of body mass was 9.5 ± 0.3 g. Figure 3.3A shows that s.c. injection of Brewer's yeast induced a significant dose-dependent decrease in the food intake compared to rats injected with saline (main effects of time ($F_{(5, 110)} = 60.70$, $P < 0.0001$), treatment ($F_{(3, 22)} = 56.93$, $P < 0.0001$) and interaction ($F_{(15, 110)} = 15$, $P < 0.0001$)). Rats receiving the high, medium or low dose of Brewer's yeast decreased food intake by approximately 60%, 50% and 25%, respectively, on the first night after injection. Food intake recovered after its initial decrease for rats injected with the high dose of Brewer's yeast but remained slightly (~ 8%) reduced on the sixth day after injection ($t = 2.5$, $P = 0.04$). Rats receiving the medium or lower dose of Brewer's yeast had a faster recovery in food intake after the initial decrease, such that, from the fourth day after injection, they were consuming similar amounts of food compared to before the injections ($t = 2.5$, $P = 0.07$ medium dose; $t = 0.16$, $P = 0.9$ low dose).

Figure 3.3B shows that the dose-dependent decrease in food intake following s.c. injection of Brewer's yeast was associated with a dose-dependent loss of body mass (main effects of time ($F_{(5, 110)} = 18.19$, $P < 0.0001$), treatment ($F_{(3, 22)} = 24.95$, $P < 0.0001$) and interaction ($F_{(15, 110)} = 3.45$, $P < 0.0001$). The body mass of rats injected with the high or medium dose of Brewer's yeast fell progressively for two days and then gradually increased, but remained stunted on the sixth day after injection ($P < 0.01$, Tukey). Over the entire six-day period following injection, rats injected with the low dose of Brewer's yeast gained a similar amount of body mass to that noted for rats injected with saline ($P > 0.05$, Tukey).

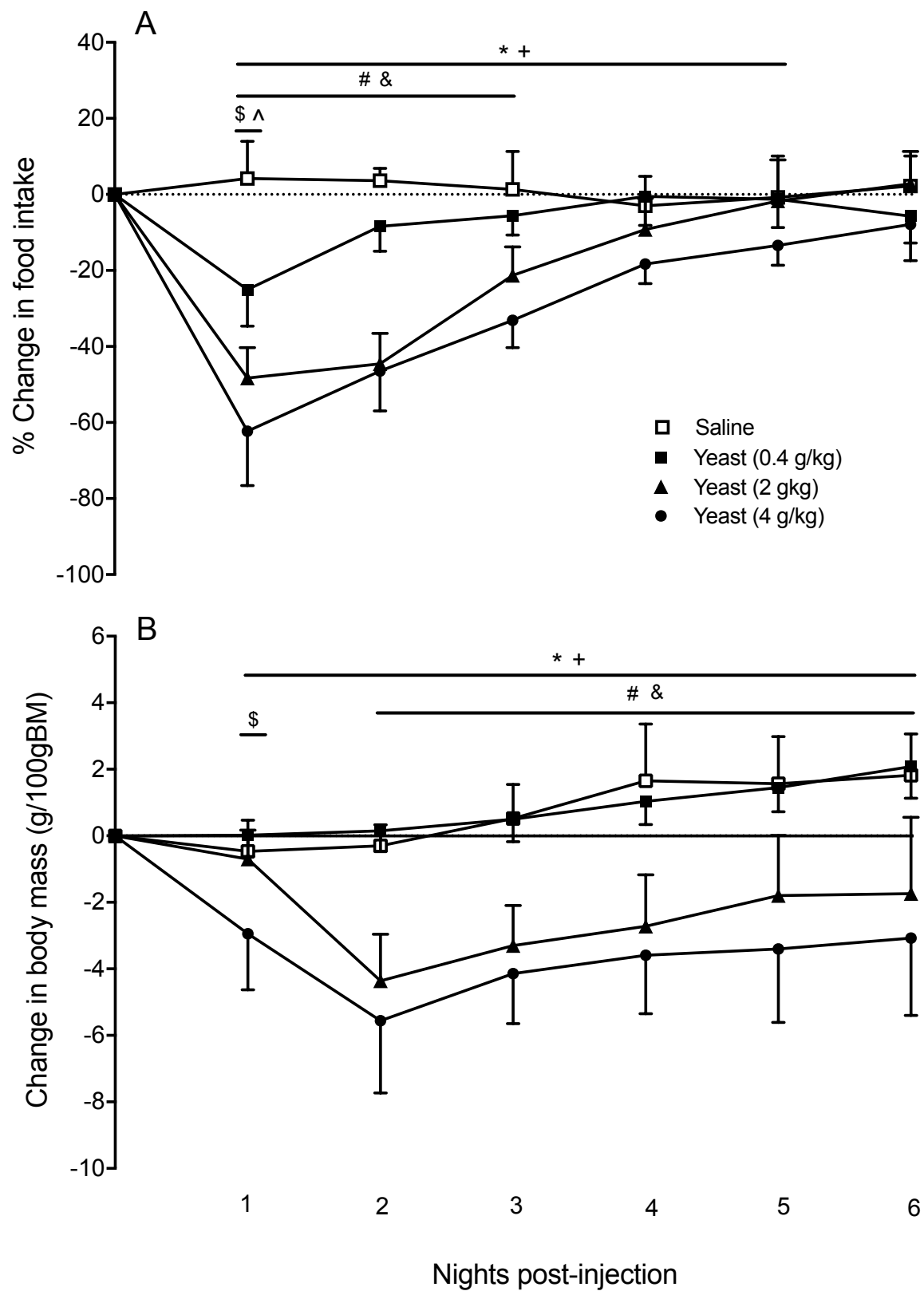


Figure 3.3: Change in food intake (A) and body mass (B) (mean and SD) of rats over six days after subcutaneous injection of either 4 g/kg Brewer's yeast (n = 9), 2 g/kg Brewer's yeast (n = 5), 0.4 g/kg Brewer's yeast (n = 6), or saline (n = 6). Food intake was calculated as grams of food consumed in 24 hours/100 g of body mass and was expressed as a percentage change from average daily food intake measured over three days before injection. Change in body mass was determined by subtracting the body mass measured on each day after the injection from the body mass measured at the same time immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass. * indicates differences between 4 g/kg and saline ($P < 0.05$, Tukey); # indicates differences between 2 g/kg Brewer's yeast and saline ($P < 0.01$, Tukey); ^ indicates differences between 0.4 g/kg Brewer's yeast and saline ($P < 0.0001$, Tukey); \$ indicates differences between 4 g/kg and 2 g/kg Brewer's yeast ($P < 0.05$, Tukey); + indicates differences between 4 g/kg and 0.4 g/kg Brewer's yeast ($P < 0.05$, Tukey); & indicates differences between 2 g/kg and 0.4 g/kg Brewer's yeast ($P < 0.05$, Tukey). One sample t test performed on each experimental group at each time point indicated that the decrease in food intake: (1) never returned to pre-injection values for rats that received 4 g/kg Brewer's yeast; (2) returned to pre-injection values on night 4 post injection for rats injected with 2 g/kg or 0.4 g/kg Brewer's yeast.

3.3.2 *Experiment 2 – Brewer's yeast induces an inflammatory response in the periphery and the brain*

3.3.2.1 *Plasma levels of pro-inflammatory cytokines*

Plasma was collected at 2, 8, 18 and 72 hours after s.c. administration of Brewer's yeast or saline. Figure 3.4A shows that s.c. injection of Brewer's yeast induced a significant dose-dependent increase in plasma levels of TNF- α compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 46.23$, $P < 0.0001$), treatment ($F_{(2, 57)} = 98.67$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 36.93$, $P < 0.0001$)). Rats injected with the high dose (4 g/kg) of Brewer's yeast had significantly elevated plasma levels of TNF- α at 2 hours and 8 hours compared to rats injected with the medium dose (2 g/kg) or saline ($P < 0.0001$, Tukey). Plasma levels of TNF- α were significantly elevated at 2 hours in rats injected with the medium dose (2 g/kg) of Brewer's yeast compared to rats injected with saline.

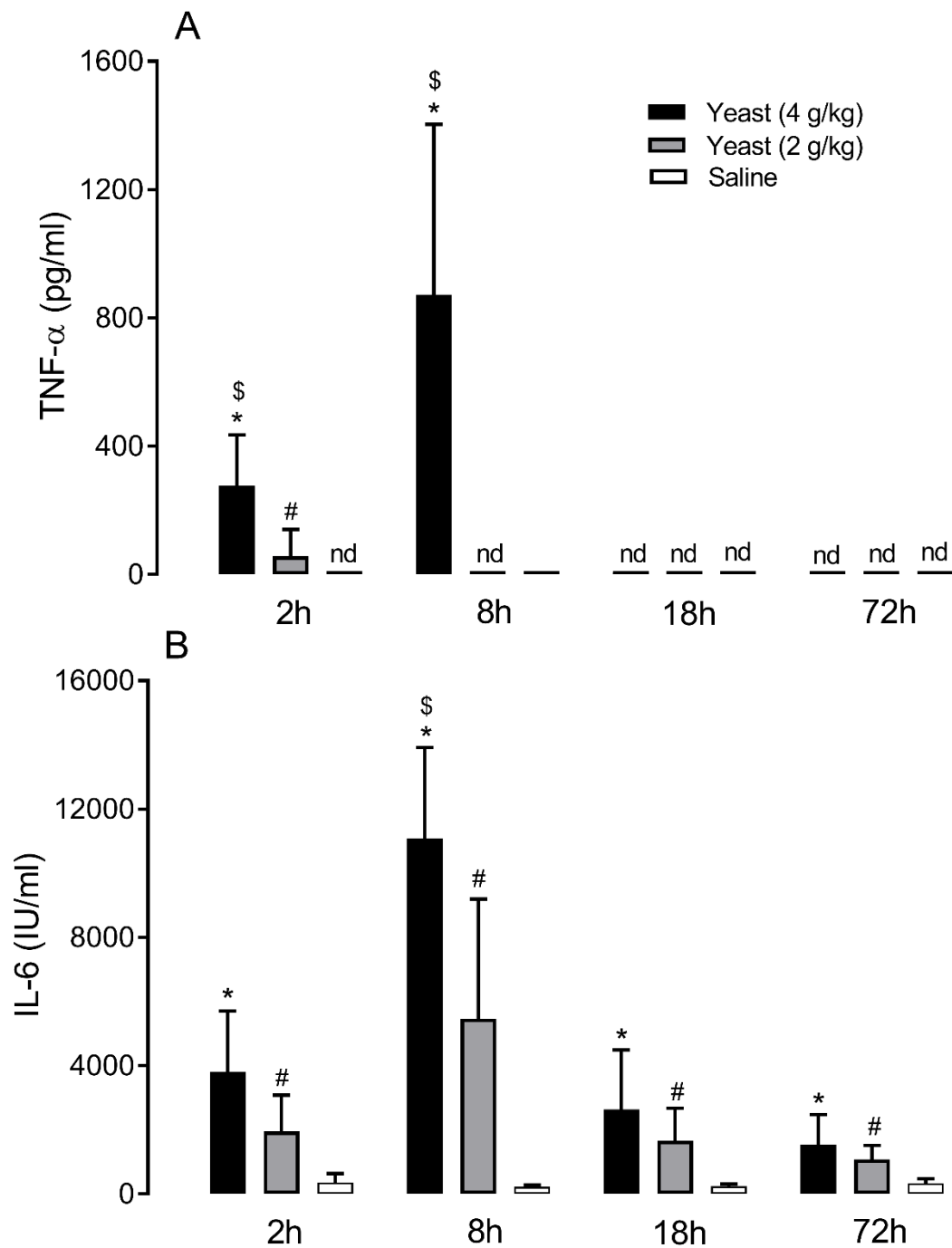


Figure 3.4: Blood plasma levels (mean and SD) of bioactive TNF- α (**A**) and IL-6 (**B**) at 2, 8, 18 or 72 hours after subcutaneous injection of either 4 g/kg Brewer's yeast, 2 g/kg Brewer's yeast or saline. Sample size per intervention group per time point = 5 - 7 rats. * Indicates differences between 4 g/kg Brewer's yeast and saline ($P < 0.01$, Tukey); # indicates differences between 2 g/kg Brewer's yeast and saline ($P < 0.05$, Tukey); \$ indicates differences between 4 g/kg and 2 g/kg Brewer's yeast ($P < 0.05$, Tukey); nd = not detectable.

Figure 3.4B shows that s.c. injection of Brewer's yeast induced a significant dose-dependent increase in plasma levels of IL-6 compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 6.60$, $P = 0.0007$), treatment ($F_{(2, 57)} = 77$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 2.92$, $P = 0.0148$)). Plasma levels of IL-6 were significantly increased at all time points (2, 8, 18 and 72 hours) in rats injected with the medium and high doses of Brewer's yeast when compared to rats injected with saline ($P < 0.05$, Tukey). At 8 hours after injection, rats that received the high dose of Brewer's yeast had significantly greater levels of plasma IL-6 than rats that received the medium dose of Brewer's yeast ($P = 0.04$, Tukey).

3.3.2.2 *Activation of NF-IL6, STAT3, NF- κ B and COX-2 in the brain*

Figure 3.5 shows representative photomicrographs of immunohistochemical detection of nuclear factor interleukin-6 (NF-IL6) in the OVLT of rats injected s.c. with the high dose of Brewer's yeast or saline. NF-IL6 immunoreactivity, which colocalised with the DNA marker DAPI, appeared in this sensory circumventricular organ as early as 2 hours after injection, was strongest at 8 hours and then decreased to reach basal levels by 72 hours after rats were injected with Brewer's yeast. Rats injected with saline did not show nuclear NF-IL6 immunoreactivity in the OVLT at any time points. The nuclear NF-IL6 signals were closely associated with blood vessels in the OVLT as shown by an additional colocalisation with von Willebrand factor (vWF). Nuclear NF-IL6 immunoreactivity was also observed in blood vessels and parenchymal cells of the cortex, ependymal cells, the choroid plexus and the median eminence of rats injected with Brewer's yeast (images not shown).

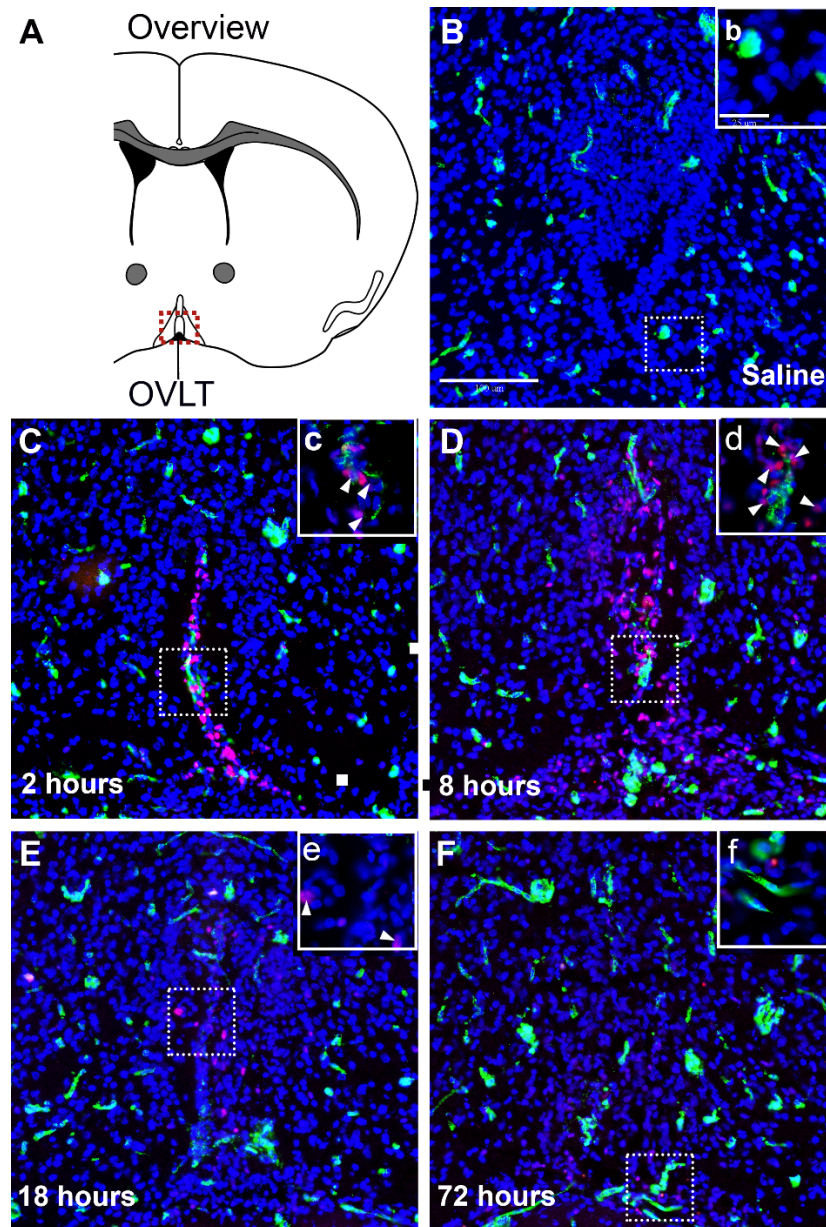


Figure 3.5: Nuclear NF-IL6 immunoreactivity in the OVLT at 2, 8, 18 and 72 hours after subcutaneous injection of either 4 g/kg Brewer's yeast or saline. (A) Schematic overview of the rat brain showing the position of the OVLT. The white arrowheads show the co-localization of red NF-IL6 signals with the nuclear DAPI stain (blue) that indicates the nuclear localization of the signals, which appeared at 2 hours (C), peaked at 8 hours (D) and decreased at 18 hours (E) and reached basal levels at 72 hours (F) after injection with Brewer's yeast. Subcutaneous injection of rats with saline was not associated with nuclear NF-IL6 immunoreactivity (B). The locations of areas used for high magnification are indicated by a dashed square in the overviews. Blood vessels were stained with von Willebrand factor (green). Scale bars: in B represents 100 μ m and applies to 20 x magnification micrographs (B-E); in b represents 25 μ m and applies to 40 x magnification inserts (b-e).

Figure 3.6A and 3.6B shows representative high-magnification photomicrographs of immunohistochemical detection of signal transducer and activator of transcription 3 (STAT3) activation in the OVLT of rats injected with the high dose of Brewer's yeast or saline. The strongest STAT3 immunoreactivity that colocalised with the DNA marker DAPI, appeared in the OVLT at 2 hours after injection and could still be detected at 18 hours, but not 72 hours, after rats were injected with Brewer's yeast. Rats injected with saline did not show nuclear STAT3 immunoreactivity in the OVLT at any of the four time points. The nuclear STAT3 signals were closely associated with blood vessels in the OVLT as shown by an additional colocalisation with vWF. Nuclear STAT3 immunoreactivity was also observed in blood vessels of the cortex (images not shown). Figure 3.6C and 3.6D shows representative high magnification photomicrographs of immunohistochemical detection of nuclear factor- κ B (NF- κ B) in the cortex of rats injected with the high dose of Brewer's yeast or saline. The strongest NF- κ B staining, which colocalised with the nucleic acid marker DAPI, appeared in the cortex at 2 hours after injection, but was not detected at 8, 18 and 72 hours after injection of Brewer's yeast.

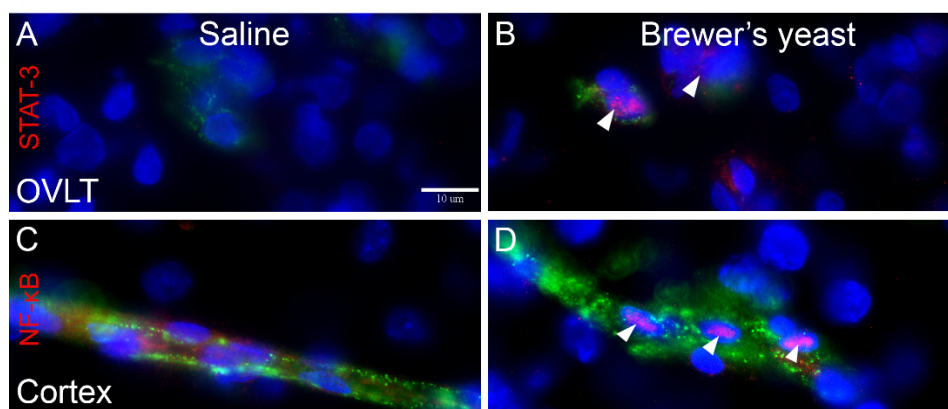


Figure 3.6: Nuclear STAT-3 immunoreactivity in the OVLT (**B**) (red shown with white arrowheads) and nuclear NF- κ B immunoreactivity in the cerebral cortex (**D**) (red shown with white arrowheads) of rats 2 hours after subcutaneous injection of either 4 g/kg Brewer's yeast or saline. Co-localization of red NF-IL6 signals with the nuclear DAPI stain (blue) indicates the nuclear localization of the STAT-3 and NF- κ B signals (red). Blood vessels were stained with von Willebrand factor (green). The nuclear STAT-3 and NF- κ B staining was associated with blood vessels, as shown by co-localization with von Willebrand factor (green). Subcutaneous injection of rats with saline was not associated with nuclear STAT-3 (**A**) or NF- κ B (**C**) immunoreactivity. Scale bar = 10 μ m and applies to all micrographs (**A-D**) which were taken at 100x magnification.

The nuclear NF- κ B signals were closely associated with blood vessels in the cortex as shown by an additional colocalisation with vWF. No nuclear NF- κ B signals were observed in the OVLT at any of the four time points in rats injected with Brewer's yeast.

Figure 3.7 shows representative high-magnification photomicrographs of immunohistochemical detection of the enzyme COX-2 in the OVLT of rats injected with the high dose of Brewer's yeast or saline. Weak signals of nuclear COX-2 immunoreactivity were detected at 2 hours, strong signals at 8 hours and 18 hours, and no signals were detected at 72 hours after Brewer's yeast injection.

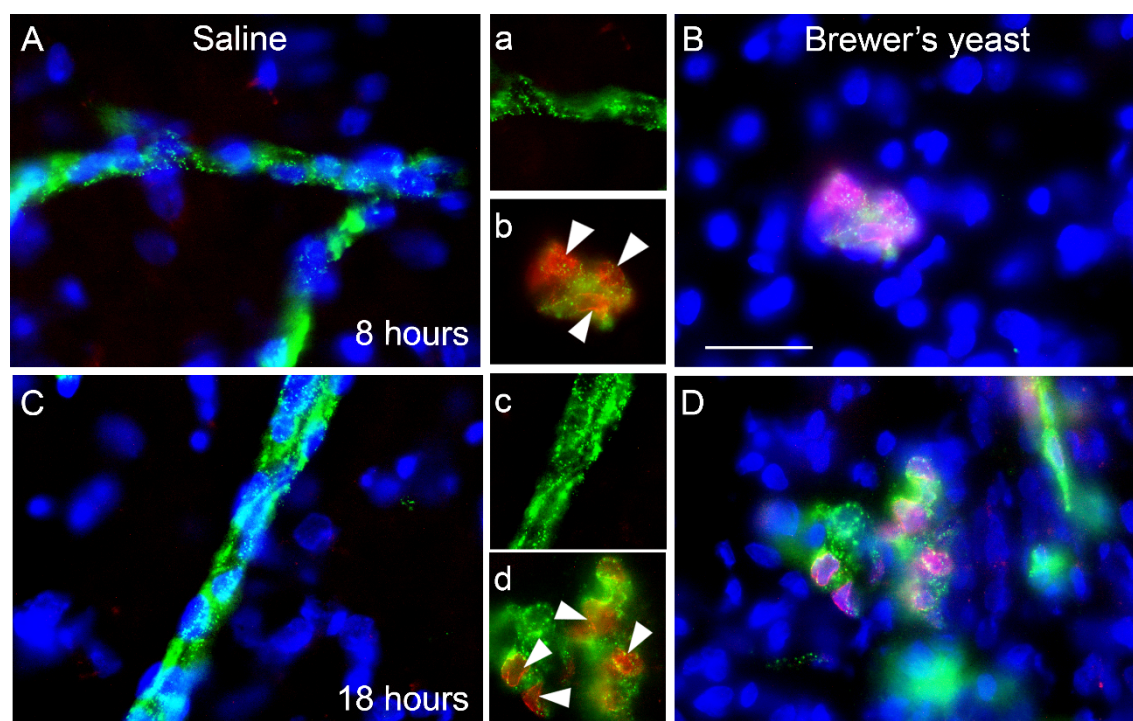


Figure 3.7: Perinuclear COX-2 immunoreactivity (red) in the OVLT at 8 hours (**B**) and 18 hours (**D**) after subcutaneous injection of either 4 g/kg Brewer's yeast or saline (**A** and **C**). Co-localization of red COX-2 signals with the nuclear DAPI stain (blue) indicates the perinuclear localization of the COX-2 signals (red). COX-2 staining (red) was associated with blood vessels as shown by co-localization with von Willebrand factor (green) (white arrowheads in inserts **b** and **d**). Subcutaneous injection of rats with saline was not associated with COX-2 immunoreactivity (**A**) and (**C**) (also see inserts **a** and **c**). Scale bar = 25 μ m and applies to all micrographs (**A** - **D**) which were taken at 100 x magnification.

The nuclear COX-2 signals co-localised with small and large blood vessels in the OVLT as shown by an additional co-localisation with vWF (Figure 3.7B and 3.7D). Nuclear COX-2 immunoreactivity was also detected in the walls of large vessels with large lumens in the subarachnoid space, lateral to the optic chiasm (images not shown). No nuclear COX-2 signals were observed in blood vessels in the OVLT at any time point in rats injected with saline.

3.3.2.3 *Relative mRNA expression of inflammatory mediators in the hypothalamus*

Figure 3.8 shows the increase in hypothalamic mRNA expression of pro-inflammatory target genes and regulatory proteins of transcription factors following Brewer's yeast injection. Figure 3.8A shows that there was a dose-dependent increase in NF-IL6 mRNA expression in the hypothalamus of rats injected with Brewer's yeast (10 ml/kg or 20 ml/kg of a 20% solution) compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 40.40$, $P < 0.0001$), treatment ($F_{(2, 57)} = 46.26$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 3.07$, $P = 0.0114$)). Rats injected with the high dose of Brewer's yeast had significantly greater hypothalamic NF-IL6 mRNA expression at 2, 8 and 18 hours compared to rats injected with the medium dose or saline ($P < 0.05$, Tukey). The upregulation of NF-IL6 mRNA in the hypothalamus was significantly greater in rats injected with the medium dose compared to rats injected with saline at 2 and 8 hours ($P < 0.05$, Tukey).

Figure 3.8B shows that there was a dose-dependent increase in the mRNA expression of the NF- κ B regulator I κ B α in the hypothalamus of rats injected with Brewer's yeast (4g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 38.55$, $P < 0.0001$), treatment ($F_{(2, 57)} = 54.62$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 8.84$, $P < 0.0001$)). Rats injected with the high or medium dose of Brewer's yeast had significantly greater hypothalamic I κ B α mRNA expression at 2, 8 and 18 hours compared to rats injected with the saline ($P < 0.05$, Tukey). The upregulation of I κ B α mRNA in the hypothalamus was significantly greater in rats injected with the high dose of Brewer's yeast compared to rats injected with medium dose of Brewer's yeast at 2 hours ($P < 0.001$, Tukey).

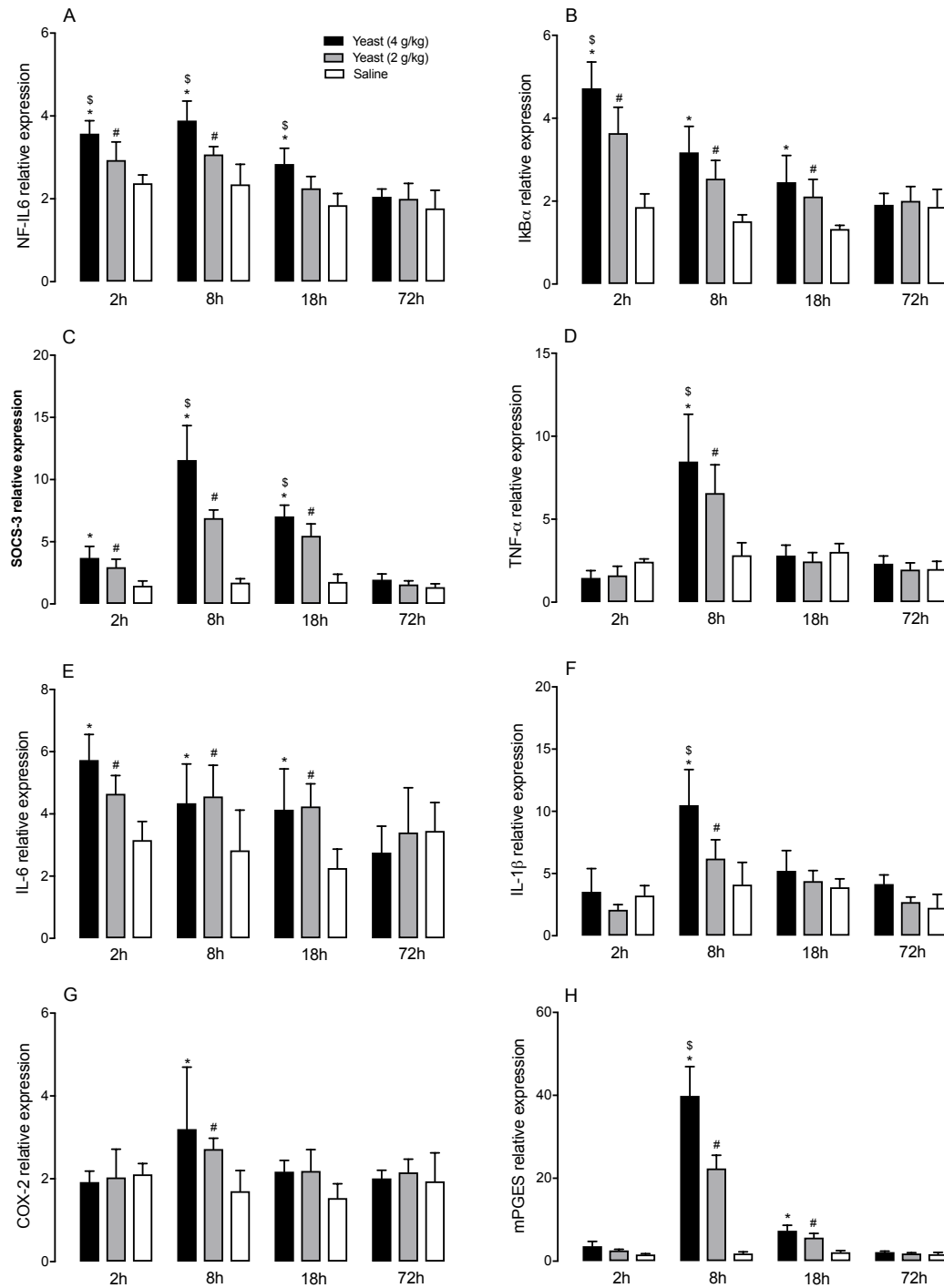


Figure 3.8: Relative mRNA expression (mean and SD) of transcription factors (A - C), pro-inflammatory cytokines (D - F) and rate limiting enzymes for PGE₂ synthesis (G - H) in the rat hypothalamus at 2, 8, 18 and 72 hours after subcutaneous injection of either 4 g/kg Brewer's yeast, 2 g/kg Brewer's yeast or 0.9% saline. Sample size per intervention group per time point = 5 - 7 rats. * Indicates differences between 4 g/kg and saline ($P < 0.05$, Tukey); # indicates differences between 2 g/kg and saline ($P < 0.05$, Tukey); \$ indicates differences between 4 g/kg and 2 g/kg ($P < 0.05$, Tukey).

Figure 3.8C shows that there was a dose-dependent increase in suppressor of cytokine signalling 3 (SOCS3) mRNA expression in the hypothalamus of rats injected with Brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 80.61$, $P < 0.0001$), treatment ($F_{(2, 57)} = 109.10$, $P < 0.0001$) and their interaction ($F_{(6, 57)} = 21.51$, $P < 0.0001$)). Rats injected with the high or medium dose of Brewer's yeast had significantly greater hypothalamic SOCS3 mRNA expression at 2, 8 and 18 hours compared to rats injected with the saline ($P < 0.05$, Tukey). The upregulation of SOCS3 mRNA in the hypothalamus was significantly greater in rats injected with the high dose of Brewer's yeast compared to rats injected with medium dose of Brewer's yeast at 8 and 18 hours ($P < 0.05$, Tukey).

Figure 3.8D and 3.8F shows that there was a dose-dependent increase in TNF- α and IL-1 β mRNA expression in the hypothalamus of rats injected with Brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (TNF- α : main effects of time ($F_{(3, 57)} = 51.94$, $P < 0.0001$), treatment ($F_{(2, 57)} = 6.82$, $P = 0.0022$) and interaction ($F_{(6, 57)} = 11.19$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 21.51$, $P < 0.0001$); IL-1 β : main effects of time ($F_{(3, 57)} = 28.90$, $P < 0.0001$), treatment ($F_{(2, 57)} = 19.71$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 5.31$, $P = 0.0002$)).

Rats injected with the high or medium dose of Brewer's yeast had significantly greater hypothalamic TNF- α and IL-1 β mRNA expression at 8 hours compared to rats injected with the saline ($P < 0.05$, Tukey). The upregulation of TNF- α and IL-1 β mRNA in the hypothalamus was significantly greater in rats injected with the high dose of Brewer's yeast compared to rats injected with medium dose of Brewer's yeast at 8 hours ($P < 0.05$, Tukey).

Figure 3.8E shows that there was an increase in IL-6 mRNA expression in the hypothalamus of rats injected with Brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 5.34$, $P = 0.0026$), treatment ($F_{(2, 57)} = 12.51$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 2.99$, $P = 0.0132$)). Rats injected with the high or medium dose of Brewer's yeast had significantly greater hypothalamic IL-6 mRNA expression at 2, 8 and 18 hours compared to rats injected with the saline ($P < 0.05$, Tukey). There were no significant differences in the upregulation of IL-6 mRNA in the hypothalamus between rats injected with the high dose of Brewer's yeast compared to rats injected with medium dose of Brewer's yeast ($P > 0.05$, Tukey).

Figure 3.8G shows that there was an increase in COX-2 mRNA expression in the hypothalamus of rats injected with Brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 3.46$, $P = 0.0221$), treatment ($F_{(2, 57)} = 4.87$, $P = 0.0111$) and their interaction ($F_{(6, 57)} = 2.28$, $P = 0.0485$). Rats injected with the high or medium dose of Brewer's yeast had significantly greater hypothalamic COX-2 mRNA expression at 8 hours compared to rats injected with the saline ($P < 0.05$, Tukey). There were no significant differences in the upregulation of COX-2 mRNA in the hypothalamus between rats injected with the high dose of Brewer's yeast compared to rats injected with medium dose of Brewer's yeast ($P > 0.05$, Tukey).

Figure 3.8H shows that there was a dose-dependent increase in mPGES mRNA expression in the hypothalamus of rats injected with Brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(3, 57)} = 250.40$, $P < 0.0001$), treatment ($F_{(2, 57)} = 130.40$, $P < 0.0001$) and their interaction ($F_{(6, 57)} = 76.69$, $P < 0.0001$)). Rats injected with the high or medium dose of Brewer's yeast had significantly greater hypothalamic mPGES mRNA expression at 8 and 18 hours compared to rats injected with the saline ($P < 0.05$, Tukey). The upregulation of mPGES mRNA in the hypothalamus was significantly greater in rats injected with the high dose of Brewer's yeast compared to rats injected with medium dose of Brewer's yeast at 8 hours ($P < 0.0001$, Tukey).

3.4 Discussion

The results from this study show that s.c. administration of Brewer's yeast dose-dependently induces fever, lethargy, anorexia and body mass stunting that is accompanied by pronounced inflammation in the periphery and the brain. To my knowledge this is the first report that fever and sickness behaviour induced by Brewer's yeast administration coincides with increased concentrations of circulating pro-inflammatory cytokines (IL-6 and TNF- α) (Figure 3.4), and enhanced mRNA and protein expression of key transcription factors and regulators (NF-IL6, STAT3, NF- κ B and SOCS3) involved in the activation or suppression of immune response genes in the brain (Figures 3.5 – 3.8). There was a pronounced increase in the mRNA expression of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and the rate-limiting enzymes for prostaglandin synthesis (COX-2 and mPGES) in the hypothalamus primarily around the peak of fever, which occurred eight hours after s.c. administration of Brewer's yeast

(Figure 3.1 and 3.8). Moreover, nuclear immunoreactivity for NF-IL6, STAT3 and COX-2 was associated with blood vessels in the OVLT. Thus, the OVLT appears to be an important communication pathway between the periphery and the brain during yeast-induced infections.

The fever and sickness behaviour observed were dose-dependent in that the high (4 g/kg) and medium dose of Brewer's yeast (2 g/kg) induced responses of greater magnitude and longer duration than did the lower dose (0.4 g/kg) (Figure 3.1 – 3.3). The fevers arising from injection of the medium and high doses of Brewer's yeast were not significantly different (time to onset ~3 hours, time to peak = 8 hours and duration 5-6 days after injection). Similarly, the lethargy induced by the high and medium dose was sufficiently intense for the rats to almost cease moving around their cages for 48 hours after injection. The rats failed to reach pre-injection activity levels over the six-day period of observation. In line with a decrease in food intake, I observed a comparable decrease in body mass between rats injected with the high dose and those injected with the medium dose of Brewer's yeast. For both doses, the decrease in body mass ultimately resulted in stunting, with the body mass failing to reach pre-injection values over the six-day period of observation. From these findings, it appears that the sickness response to injection of the medium dose is very similar to that induced by the high dose. Thus, for purposes of screening for the efficacy of antipyretic drugs against the sickness response induced by Brewer's yeast, injecting the high dose proffers no advantage over the medium dose. From observations made in my study, injection of the high dose is associated with other undesirable responses such as; the development of an initial hypothermia before fever, excessive inflammation around the head and neck region on the first night and day after injection, and a later development of an abscess: all of which do not occur when one injects the medium or the low dose.

The short-lived hypothermia that develops after injection of the high dose of Brewer's yeast is similar to that which occurs when one injects high, septic doses of bacterial endotoxin (Derijk and Berkenbosch, 1994, Leon, 2004, Ueno et al., 1982). Despite earlier arguments that hypothermia is a nonspecific, non-beneficial, unregulated secondary response to an infectious agent, recent studies have shown that the hypothermia is a tightly regulated, adaptive response, which occurs during systemic inflammation to increase chances of survival (Derijk et al., 1994b, Romanovsky and Szekely, 1998, Leon, 2004). Research has shown that following infection, the development of hypothermia is mediated through deliberate metabolic depression, peripheral vasodilation and cold-seeking behaviour (Corrigan, et al., 2014,

Romanovsky and Szekely, 1998). Environmental conditions, in particular ambient temperatures are also believed to determine whether rodents challenged with high doses of a pyrogen develop hypothermia or a fever (Derijk et al., 1994a, Rudaya et al., 2005, Liu et al., 2012). My experiments were carried out at sub-thermoneutral temperatures (22 ± 2 °C) and this may also explain the development of the hypothermia in rats injected with the high dose of Brewer's yeast.

Published pharmacological antipyretic drug testing guidelines recommend that the test compound or the standard drug be administered 18 hours after injection of yeast and temperatures be recorded at time intervals of 30 – 60 min for five hours thereafter (Thangaraj, 2016, Vogel, 2002). In this study, Brewer's yeast was injected two hours before lights off and 18 - 23 hours after injection corresponded to the plateau phase of the fever. During the plateau phase of the fever, abdominal temperature of rats receiving the high and medium doses of Brewer's yeast was consistently greater ($1 - 1.5$ °C) than the abdominal temperature of rats that received saline. The increased body temperature noted during the plateau phase of the fever supports the recommendation of antipyretic administration taking place 18 hours after Brewer's yeast administration and shows that screening for antipyretic effects can continue beyond 23 hours. However, it is important to note that the fever was prominent only during the lights-on phase and masked during the lights-off phase due to the nycthemeral temperature rhythm of rats.

In addition to fully characterizing the yeast-induced fever-curve and sickness behaviours across a range of Brewer's yeast doses commonly used in antipyretic screening in rats, I also characterised the spatiotemporal activation of inflammatory mediators in the periphery and brain. The time-course of the inflammatory changes showed a marked increase in the synthesis of pro-inflammatory cytokines, key prostaglandin-synthesizing enzymes and inflammatory transcription factors in the periphery and brain between 2 and 18 hours after Brewer's yeast administration. Overall, the most pronounced increase in inflammatory mediators was noted eight hours after Brewer's yeast injection, with a muted response at 18 hours (Figures 3.4 and 3.8). Thus, studies wanting to explore the mechanism of action of antipyretics may consider administering the antipyretic drug at 8 hours after Brewer's yeast administration as it corresponds to a time when there are higher levels of inflammatory markers in the periphery and in the brain.

The fever, lethargy, anorexia and body mass stunting induced by s.c. administration of Brewer's yeast was accompanied by an increase in plasma levels and hypothalamic mRNA expression of the pro-inflammatory cytokines IL-6 and TNF- α (Figure 3.4 and 3.8). A key role for peripherally-released and centrally-released IL-6 in mediating fever has been suggested based on the findings that injecting recombinant IL-6 into the brains of rats induces fever (Harden et al., 2008, LeMay et al., 1990, Dinarello et al., 1991b) and that neutralizing the action of IL-6 in the periphery, using a species-specific IL-6 antiserum, completely prevented fever from occurring following localized administration of LPS (Cartmell et al., 2000, Harden et al., 2006, Rummel et al., 2006). In this study, increased plasma concentrations of bioactive IL-6 and increased hypothalamic IL-6 mRNA expression were observed from as early as 2 hours injection of the medium and high doses of Brewer's yeast, suggesting that IL-6 may be involved in the onset of Brewer's yeast induced fever. Although hypothalamic expression of IL-6 mRNA had returned to basal levels by 72 hours after injection, circulating levels of IL-6 were still significantly higher in rats injected with Brewer's yeast suggesting that IL-6 maybe responsible for the maintenance of fever beyond 72 hours.

In line with the increase in IL-6 an increase in plasma concentrations and hypothalamic mRNA expression of TNF- α was also noted. TNF- α production mirrored the peak of the fever response. Findings supporting a pyrogenic action for peripherally and centrally-released TNF- α include reports that LPS-induced fever in rabbits and guinea-pigs is attenuated when the action of TNF- α is inhibited (Kawasaki et al., 1989, Mabika and Laburn, 1999, Roth et al., 1998) and injecting recombinant TNF- α into the brains of rabbits and mice induces fever (Sundgren-Andersson et al., 1998, Kapas et al., 1992).

The synthesis of the pro-inflammatory cytokines and prostaglandins proposed to be involved in mediating fever and sickness behaviour occurs via activation of inflammatory transcription factors, such as NF-IL6, STAT3 and NF- κ B [for review see Rummel (2016)]. Following activation, transcription factors translocate from the cytoplasm into the nucleus where they regulate the expression of inflammatory target genes identified as important mediators of fever and sickness behaviour [for review see Roth and Blatteis (2014), Pecchi et al. (2009)]. Using immunohistochemical staining and real-time PCR, I examined the mRNA expression, activation and translocation of the transcription factors in the brains of rats injected with

Brewer's yeast. While the signals were observed in several structures in the brain namely the cerebral cortex, choroid plexus, ependymal cells and median eminence, the main areas of focus for my study were the hypothalamus and the OVLT. The OVLT expresses low basal levels of nuclear NF-IL6 and STAT3 (Nadeau et al., 2005, Rummel et al., 2004), but activation and nuclear translocation of these transcription factors increases dramatically during inflammation induced by LPS administration (Damm et al., 2011, Rummel et al., 2004, Voss et al., 2007, Rummel et al., 2005). Immunohistochemical staining for nuclear translocation and activation of transcription factors in this sensory CVO therefore serves as a useful tool for investigating yeast-related inflammatory signalling between the periphery and the brain.

A clear, time-dependent activation of NF-IL6 was observed in the OVLT following injection of Brewer's yeast (see Figure 3.5). Others, using different inflammatory stimuli, have reported similar findings (Damm et al., 2011, Damm et al., 2012, Straccia et al., 2013). The spatiotemporal appearance of NF-IL6 in Brewer's yeast-induced inflammation is similar to the activation resulting from LPS administration i.e. an early onset (in this case 2 hours), and a late peak at 8 hours (Damm et al., 2011). The activation of STAT3 was also observed in endothelial cells of the OVLT (see Figure 3.6) following Brewer's yeast administration. Immunoreactive STAT3 signals were closely associated with blood vessels and STAT3 activation showed an early onset (peak at 2 hours) although it remained significantly high for up to 18 hours after injection. STAT3 activation has been implicated in the expression of SOCS3, which mediates feedback inhibition of cytokine signalling pathways (Alexander, 2002). Hypothalamic mRNA expression of SOCS3 was increased between 2 and 18 hours after s.c. administration of Brewer's yeast (Figure 3.8).

Endothelial STAT3 activation has been shown to play an indispensable role in COX-2 induction and PG synthesis and specific deletion of endothelial STAT3 attenuated the COX-2 production and the febrile response induced by a moderate immune challenge to LPS (Eskilsson et al., 2014). The appearance of COX-2 immunoreactivity in the blood vessels preceded the development of the fever, suggesting that this enzyme is involved in PGE₂ synthesis during the early phases of Brewer's yeast induced fever. Although I did not stain for PGE₂ synthase in the blood vessels to ascertain that the product of COX-2 activity (PGH₂) is indeed converted to PGE₂, I used RT-PCR to show the upregulation of mPGES gene expression in the hypothalamus. In the absence of the inflammatory stimulus, mPGES synthase expression

in the hypothalamus was minimal, but a dramatic (40-fold) increase was observed corresponding to the peak of Brewer's yeast induced fever (see Figure 3.8H).

Although there was no NF- κ B immunoreactivity observed in the OVLT, it is interesting to note that the expression of its regulatory inhibitory molecule I κ B α , which usually matches its activation patterns (Quan et al., 1997), was significantly raised in the hypothalamus and peaked at the earliest time-point (2 hours). NF- κ B activation in the OVLT has been shown to occur as early as 30 min after peripheral injection of IL-1 β (Nadjar et al., 2003) and thus it is possible that the earliest time point used in this study (2 hours) was rather late to detect its nuclear translocation in the OVLT. NF- κ B translocation was however observed in blood vessels of the cortex (see Figure 3.6D).

Overall the cascade of cytokine release in the periphery explains the activation of inflammatory transcription factors in the brain following Brewer's yeast administration. To varying degrees the activation of NF-IL6, STAT3 and NF- κ B in the hypothalamus and OVLT appear to be involved in the initiation and maintenance, and SOCS3 in the termination of the brain inflammatory and sickness response to yeast. The sickness response to Brewer's yeast remained long after the levels of cytokines and rate limiting enzymes for PGE₂ synthesis (e.g. COX-2) had returned to basal levels. There is a possibility that direct neural afferents (e.g. vagal and other cutaneous afferents) could be responsible for sending immunosensory information from the periphery to the brain without need for humoral signals [see Romanovsky (2000), Quan and Banks (2007) and Dantzer et al. (2000)]. The abscess formed on the site of Brewer's yeast injection, could therefore act as a reservoir for immunosensory signals carried by neural afferents to the brain. It is also possible that other variants of the COX enzyme e.g. COX-1 and COX-3 could possibly be involved in mediating the longer-lasting aspects of the Brewer's yeast-induced sickness response (Chandrasekharan et al., 2002).

In conclusion, from the results obtained in this study, I have established that Brewer's yeast induces a high magnitude and long-lasting sickness response that is characterised by an initial hypothermia (at high doses), fever, anorexia, lethargy and growth-stunting. The recommended 18th hour post yeast injection is an appropriate time point for measuring effects of a drug on thermal responses as it corresponds with the plateau phase of fever when temperatures are consistently increased and not changing. Mechanistically however, there are more

inflammatory mediators present in blood and in the brain at 8 hours and this time point may be a more appropriate one to intervene for studies investigating the mechanisms of action of the antipyretic drugs. At the recommended dose of 4 g/kg, Brewer's yeast injection is associated with the development of an abscess and excessive inflammation. Injection of the medium dose induces a sickness response of similar magnitude to that induced by the high dose. In the next chapter I therefore I explore the possibility of using zymosan (a cell wall component of Brewer's yeast) as an alternative pyrogen for antipyretic drug testing and elucidation of the mechanism of action of test drugs.

3.5 References

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CHAPTER FOUR

Pyrogenic models used during antipyretic testing: The potential use of zymosan to induce fever and sickness behaviour during antipyretic testing

Data and ideas from this chapter have been written up as a scientific paper and submitted to *Acta Physiologica*. Dangarembizi, R., Rummel, C., Roth, J., Erlwanger, K H., Madziva, M T and Harden, L M. Zymosan: an inducer of fever and sickness behaviour through activation of inflammatory mediators in the brain.

4.1 Introduction

As outlined in **Chapter 1** and **Chapter 3** of this thesis, *Saccharomyces cerevisiae* (Brewer's yeast) has, for close to a century, remained the recommended pyrogen for inducing fever in pharmacological, especially ethnopharmacological, studies screening antipyretics in rats (Vogel, 2002, Thangaraj, 2016). However, the large volumes and doses of live yeast administered to rats in these pharmacological studies are associated with the development of an abscess (Todd, 1986), a prolonged sickness response e.g. lethargy, anorexia and body mass stunting (see Figures 3.1 - 3.3 earlier in this thesis). As mentioned earlier in the introductory chapter, the animal research ethics welfare principle of refinement, states that procedures and techniques used for collecting data on experimental animals should cause minimal suffering and pain (Fenwick et al., 2009). Owing to the animal welfare challenges associated with using live yeast as a pyrogen, there is a need to find an alternative pyrogen that can induce reasonably long fevers that allow for pharmacological manipulations without causing unnecessary pain and suffering to the experimental animals. LPS, a bacterial pyrogen has been suggested as an alternative pyrogen but no studies have explored the possibility of using zymosan as an alternative for simulating fungal infections in rodents.

Zymosan, an immunogenic cell wall extract of *S. cerevisiae*, is made up of mainly β -glucans and mannans and has been shown to stimulate an immune response through activation of toll like receptor 2/6 (TLR2/6) and Dectin 1 (Brown et al., 2003, Gantner et al., 2003, Dillon et al., 2006). Owing to its immunostimulatory effects, zymosan has been used to induce arthritis (Frasnelli et al., 2005, Gegout et al., 1994, Keystone et al., 1977), pleurisy (Utsunomiya et al., 1998, Tarayre et al., 1989), peritonitis (Cash et al., 2009, Sakashita et al., 2000), oedema (Erdő et al., 1993), shock (Asmuth et al., 1990b, Volman et al., 2005) and other inflammatory diseases in experimental animals.

Intra-articular injection of zymosan at doses between of 4 - 10 mg/kg has been shown to induce fever, anorexia and lethargy, albeit short-lived (lasting only up to 4 hours), (Gegout et al., 1994, Kanashiro et al., 2009). While these studies may provide empirical evidence that zymosan administration can successfully induce fever and sickness behaviour, the low doses of zymosan used in these studies induce very short-lived fevers that are undesirable for antipyretic screening. Intraperitoneal injection of greater doses of zymosan (500 - 1000 mg/kg) on the other hand induced hypothermia, shock, multiple organ failure and increased mortality

(Fantuzzi et al., 1997, Volman et al., 2005). Thus, in order to test the suitability of zymosan as an alternative/additional pyrogen in antipyretic drug testing, there remains a need to identify a dose and route of administration that will induce reasonably long, stable fevers and inflammation.

As discussed previously in **Chapter 3**, the knowledge of the inflammatory mediators activated by an experimental pyrogen is important for determining the efficacy and mechanism of action of antipyretic drugs. Previous studies have reported that zymosan-induced inflammation is mediated by pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α (Fantuzzi et al., 1997, Bastos-Pereira et al., 2014) which ultimately induce prostaglandin E₂ (PGE₂) synthesis in the brain (Lima et al., 2016). Using *in vitro* techniques, others further showed that zymosan could activate neuroglial cells cultured from the febrigenic regions of the brain, the OVLT and the MnPO, to release IL-6 and TNF- α (Bastos-Pereira et al., 2014). Although these studies provide the first evidence that these mediators are stimulated during zymosan-induced inflammation, a spatiotemporal description of their activation would be more valuable for determination of mechanisms during antipyretic screening. Additionally, there are no studies describing the activation of the prominent inflammatory transcription factors (NF- κ B, NF-IL6 and STAT-3), their target genes or rate-limiting enzymes for PGE₂ synthesis (COX-2 and mPGES) in the brain after zymosan administration. Thus, our knowledge of the inflammatory pathways subserving the zymosan-induced sickness response remains incomplete.

I therefore carried out this study to determine if subcutaneous injection of zymosan can successfully induce fever and a sickness response that can be used in pharmacological antipyretic drug testing. I also sought to investigate the neuroinflammatory pathways and mediators that are activated during the zymosan-induced sickness response. I monitored body temperature, nocturnal cage activity, food intake and body mass gain as indicators of sickness. To establish if zymosan activates the same suite of inflammatory mediators as Brewer's yeast, I measured circulating levels of cytokines and assessed the activation of the key inflammatory transcription factors NF- κ B, NF-IL6 and STAT-3 in the OVLT. I also measured the expression of the target genes for these transcription factors (cytokines, COX-2, mPGES and other inflammatory regulators) in the hypothalamus. My results show that subcutaneous injection of 300 mg/kg zymosan induced fever, lethargy, anorexia and growth-stunting. The sickness

response was accompanied by an increase in circulating levels of pro-inflammatory cytokines, activation of inflammatory transcription factors and an increase in the expression of cytokines and key prostaglandin-synthesizing enzymes in the brain.

4.2 Methods

4.2.1 Animals

Male Sprague Dawley rats were purchased from the National Health Laboratory Service (Johannesburg, South Africa) and housed in the Central Animal Service Unit of the University of the Witwatersrand Johannesburg under conditions previously described in section 3.2.1. On the day of injection of the pyrogen, rats had a body mass of between 250 and 300 g. All experiments were performed in accordance with the guidelines of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Screening Committee (ethics number 2014/10/C see Appendix 1).

4.2.2 Pyrogen administration

Rats received a single subcutaneous (s.c.) injection of Zymosan A from *Saccharomyces cerevisiae* (Sigma Aldrich, St Louis, USA: Lot number BCBP2641V) reconstituted in a vehicle of 0.9% sterile, non-pyrogenic saline (Sabax, Johannesburg, South Africa). To make the zymosan solution, 3 g of powdered zymosan was dissolved with 100 ml of saline and vortexed until there were no lumps. The zymosan was incubated in a water bath at 37°C for 10 mins before being ultrasonicated for a further 2 minutes, in 30-second bouts. The zymosan solution was injected in the dorsum of the neck at a dose of either 300 mg/kg or 30 mg/kg.

4.2.3 Body temperature and cage activity

As previously described by Hopwood et al. (2009), core body temperature and cage activity of rats was measured using sterile, wax-coated temperature-sensitive radiotelemeters (TA10TA-F40, Data Sciences, St Paul, MN, USA), that had been implanted intra-abdominally, with rats

under general anaesthesia (as described fully in section 3.2.3). The rats were given a week to recover from surgery.

4.2.4 *Body mass and food intake*

Body mass and food intake were measured daily, using an electronic scale (Diamond, US) accurate to 1 g, two hours before the beginning of the dark phase of the circadian cycle. To measure food intake, 200 g of standard rat chow were given to each rat and the daily intake was the difference between the initial weight of food at the start (200 g) and the food remaining after 24 hours.

4.2.5 *IL-6 and TNF- α bioassays*

To determine the level of IL-6 and TNF- α in plasma, a bioassay based on the dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line (Aarden et al., 1987) and on the cytotoxic effect of TNF on a mouse fibrosarcoma cell line WEHI 164 subclone 13 (Espevik and Nissen-Meyer, 1986) was performed as previously described in section 3.2.5.

4.2.6 *Tissue preparation, immunohistochemistry and RT-PCR*

Coronal brain sections were prepared from areas encompassing the vascular organ of the lamina terminalis (OVLT) as described fully in section 3.2.6. Double immunohistochemistry was performed as described in section 3.2.7 to measure activation of NF-IL6, STAT3 and COX-2, and to determine the contribution of endothelial cells to the immunoreactive cell population. Details of primary and secondary antibody dilutions are as described in Table 3.1. Processed sections were analysed using fluorescence microscopy as described previously in section 3.2.8.

Hypothalamic mRNA expression of NF-IL6, I κ B α , SOCS3, COX-2, mPGES, TNF- α , IL-1 β , and IL-6 was measured using RT-PCR. The procedure for RT-PCR and the assay IDs for the genes assayed in this study are as described in section 3.2.9.

4.2.7 Experimental procedures

4.2.7.1 Experiment 1: Characterisation of fever and sickness behaviour

Rats were assigned randomly to receive one of three treatments; 300 mg/kg zymosan (n=8), 30 mg/kg zymosan (n=7) or saline (n=7). All substances were injected at a volume of 10 ml/kg body weight. I administered the injections at 17:00, one hour before lights off at 18:00. Core body temperature, cage activity, food intake and body mass of the rats were monitored for three days before and one day after the s.c. injection of zymosan.

4.2.7.2 Experiment 2: Spatiotemporal characterisation of inflammatory mediators

Separate groups of rats were randomly assigned to receive 300 mg/kg zymosan or saline, before brain tissue was collected under terminal anaesthesia induced with an i.p. injection of 1 ml sodium pentobarbital (Euthanase, 200 mg/ml; Kyron Laboratories (Pty) Ltd., South Africa). Blood and brain samples were collected at: 2, 8 and 18 hours after injection; with five to seven rats euthanased per time point for each of the intervention groups. These three time points were chosen to represent three different phases of the febrile response; the early phase (2 hours), the early plateau phase (8 hours) and the late plateau phase (18 hours). Blood was collected by cardiac puncture into EDTA tubes after which the rats were transcardially perfused using ice cold saline (0.9%) for 2 min. Whole brains were recovered and quickly frozen on powdered dry-ice and stored at -80 °C.

4.2.8 Data analysis

All data are expressed as mean and standard deviation (SD) and were analysed using GraphPad Prism (version 7.0, GraphPad Software Inc, USA). Significance was set at $P < 0.05$. For statistical purposes, the original five-minute temperature recordings were averaged over successive two-hour periods. The two-hour temperature means were analysed by two-way analysis of variance (ANOVA) with treatment and time as main effects. A Bonferroni *post hoc* test was used to identify differences between groups whenever the ANOVA detected significant main effects or interactions. Abdominal temperature responses were plotted as abdominal temperature-time curves in 30-min intervals. The five-minute nocturnal activity

counts for each rat were summed for 12 hours (18:00 – 06:00) and the summed values were averaged for three nights before the day of injection, when the rats were undisturbed in their home cages. Change in nocturnal cage activity after injection was calculated as the difference between the night-time activity following injection (18:00 – 06:00) and the average night-time activity in the three nights before injection, when the rats were undisturbed in their home cages, and expressed as a percentage of the average pre-injection activity. Food intake post injection was calculated as grams of food consumed in 24 hours per 100 g of body mass and expressed as a percentage change from the mean daily food intake measured over three days before injection.

Twenty-four-hour change in body mass (g) was determined by subtracting the body mass measured at 24 hours after the injection from the body mass measured immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass. Changes in nocturnal cage activity, food intake and body mass were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. To determine whether the change in cage activity, food intake and body mass after injection was significantly different from pre-injection values, a one-sample t-test was performed for each of the experimental groups. Cytokine levels and relative mRNA expression across treatments at each of the 3 time points (2, 8 and 18 hours after injection) were compared by two-way ANOVA with treatment and time as main effects. We used a Bonferroni *post hoc* test to identify differences between groups when the ANOVA detected significant main effects or interactions. Where cytokine levels were undetectable, samples were assigned a value equivalent to the detection limit of the assay. Plasma cytokine data that were not normally distributed were log-transformed prior to statistical analysis. The data presented are non-transformed means and SD, but the associated significance values were derived from log-transformed data.

4.3 Results

4.3.1 Experiment 1 - Zymosan induces fever, lethargy, anorexia and body mass stunting

4.3.1.1 Body temperature and nocturnal activity

Figure 4.1 shows the core body temperature recorded over one day for rats that received s.c. injections of either 300 mg/kg zymosan, 30 mg/kg zymosan or saline. The main effects of time ($F_{(13, 247)} = 95.25$, $P < 0.0001$), treatment ($F_{(2, 19)} = 124.40$, $P < 0.0001$) and interaction ($F_{(26,$

$_{247}) = 40.41$, $P < 0.0001$) were significant such that rats injected with zymosan developed a fever while rats injected with saline did not. Body temperatures of rats injected with the high dose of zymosan (300 mg/kg) started to increase after a latent period of ~ 2 hours and peaked ($T_{\max} 38.95 \pm 0.23$ °C) ~ 9 hours after injection.

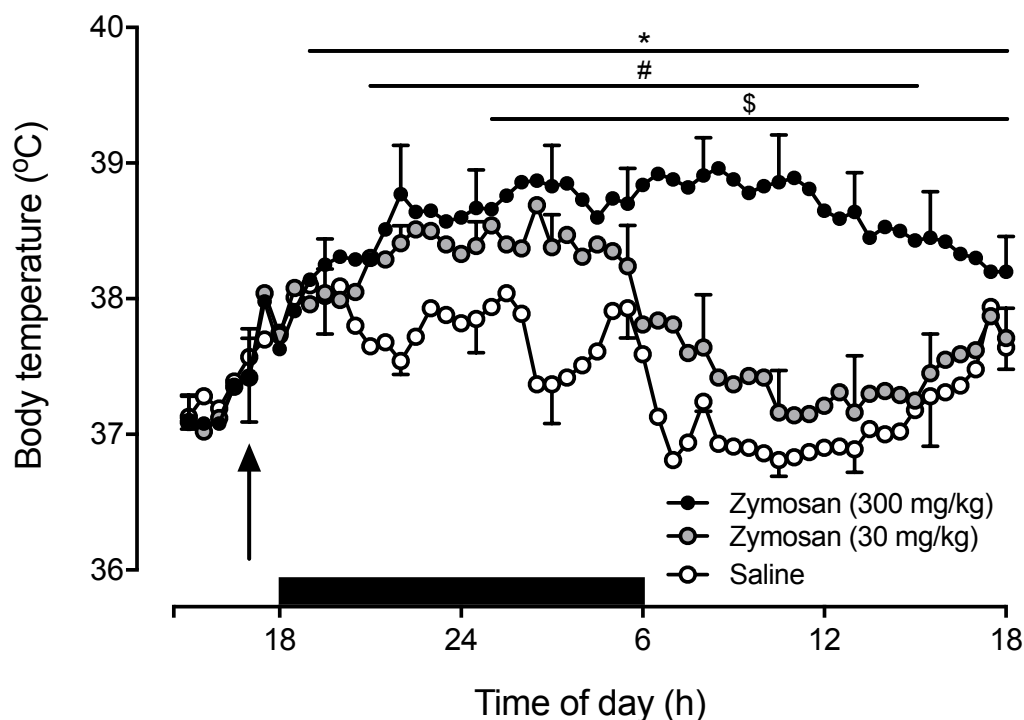


Figure 4.1: Core body temperature (mean and SD) of rats over one day following subcutaneous injection of either 300 mg/kg zymosan ($n = 8$), 30 mg/kg zymosan ($n = 7$) or saline ($n = 7$). Core body temperatures plotted in 30-minute intervals. The black arrow indicates injection time and black bars indicate lights off (18:00 - 06:00 clock time). * indicates differences between 300 mg/kg zymosan and saline ($P < 0.01$, Bonferroni), # indicates differences between 30 mg/kg zymosan and saline ($P < 0.05$, Bonferroni). \$ indicates differences between 300 mg/kg zymosan and 30 mg/kg zymosan ($P < 0.01$, Bonferroni).

The body temperature of rats injected with the lower dose (30 mg/kg) doses of zymosan started to increase after a latent period of ~ 4 hours and peaked ($T_{\max} 38.56 \pm 0.25$ °C) ~ 6 hours after injection. Fever induced by the 300 mg/kg dose of zymosan persisted for the first night and day after injection ($P < 0.01$, Bonferroni), while fever induced by the lower dose (30 mg/kg) persisted for 18 hours after injection ($P < 0.05$, Bonferroni). The increase in body temperature induced by the high dose of zymosan was greater than that induced by the lower dose ($P < 0.01$, Bonferroni).

Figure 4.2 shows that s.c. injection of zymosan and saline both induced a significant decrease in nocturnal cage activity over 12 hours after injection, but the decrease following zymosan injection (300 mg/kg; $59.4 \pm 10.6\%$ and 30 mg/kg; $37.9 \pm 13.1\%$) was significantly greater than that following saline injection ($7.3 \pm 6.6\%$) (one-way ANOVA, $F_{(2, 19)} = 46.58$, $P < 0.0001$). The magnitude of the decrease in nocturnal cage activity was dependent on the dose of zymosan injected as rats that had received 300 mg/kg decreased nocturnal cage activity by $\sim 20\%$ more than rats that had received 30 mg/kg ($P < 0.01$, Bonferroni). The mean change in nocturnal cage activity of rats receiving the vehicle was not significantly different from zero ($t = 1.65$, $P = 0.15$). Injection of saline therefore did not have any effect on nocturnal cage activity of rats.

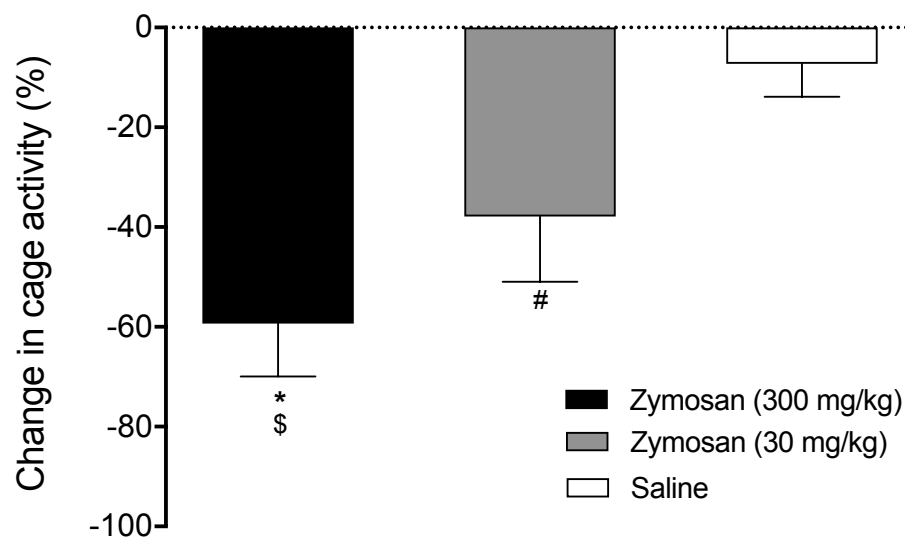


Figure 4.2: Percentage change in nocturnal cage activity (mean and SD) of rats over 12 hours (18:00 – 06:00) after subcutaneous injection of either 300 mg/kg zymosan ($n = 8$), 30 mg/kg zymosan ($n = 7$) or saline ($n = 7$). * indicates differences between 300 mg/kg zymosan and saline ($P < 0.0001$, Bonferroni), # indicates differences between 30 mg/kg zymosan compared to saline ($P < 0.0001$, Bonferroni), \$ indicates differences between 300 mg/kg and 30 mg/kg zymosan ($P < 0.01$, Bonferroni). A one sample t test indicated that cage activity decreased significantly from pre-injection values in all groups (300 mg/kg $t = 15.9$ and $P < 0.0001$; 30 mg/kg $t = 7.7$ and $P = 0.0003$; saline $t = 2.9$ and $P = 0.0274$).

4.3.1.2 Food intake and body mass

For the three days before receiving an injection of zymosan or saline, the average daily food consumption of the rats per 100 g of body mass was 7.5 ± 0.3 g. Figure 4.3A shows that s.c.

injection of 300 mg/kg zymosan induced a significant decrease ($37.9 \pm 7.7\%$) in 24-hour food intake of rats compared to injection of saline (one-way ANOVA, ($F_{(2, 19)} = 45.09$, $P < 0.0001$). The food intake of rats that had received 30 mg/kg zymosan or saline was significantly different to the food intake of rats that had received 300 mg/kg, but it was not significantly different to pre-injection values ($t = 0.79$, $P = 0.46$ and $t = 0.67$, $P = 0.52$). Thus, the decrease in food intake induced by zymosan injection was dependent on the dose administered.

Figure 4.3B shows that the decrease in food intake following s.c. injection of the 300 mg/kg of zymosan was associated with a significant loss of body mass (one-way ANOVA, $F_{(2, 19)} = 12.54$, $P = 0.0003$). Over the 24 hour period after injection there was no significant change in the body mass of rats that received 30 mg/kg zymosan ($t = 1.2$, $P = 0.287$), while rats that received saline significantly gained (1.1 ± 0.8 g) body mass ($t = 3.3$, $P = 0.0156$). Thus, the loss of body mass induced by zymosan injection was dependent on the dose administered.

4.3.2 Experiment 2 – Zymosan induces an inflammatory response in the periphery and the brain

4.3.2.1 Plasma levels of pro-inflammatory cytokines

Blood plasma was collected at 2, 8 and 18 hours after s.c. administration of 300 mg/kg zymosan or saline. Figure 4.4A shows that rats injected with zymosan had significantly elevated plasma levels of TNF- α at 2 hours after injection compared to rats injected with saline (main effects of time ($F_{(2, 23)} = 20.42$, $P < 0.0001$), treatment ($F_{(1, 23)} = 30.64$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 20.42$, $P < 0.0001$)). Figure 4.4B shows that rats injected with zymosan had significantly elevated plasma levels of IL-6 at 2, 8 and 18 hours compared to rats injected with saline (main effects of time ($F_{(2, 23)} = 2.43$, $P = 0.11$), treatment ($F_{(1, 23)} = 189.70$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 0.69$, $P = 0.69$)).

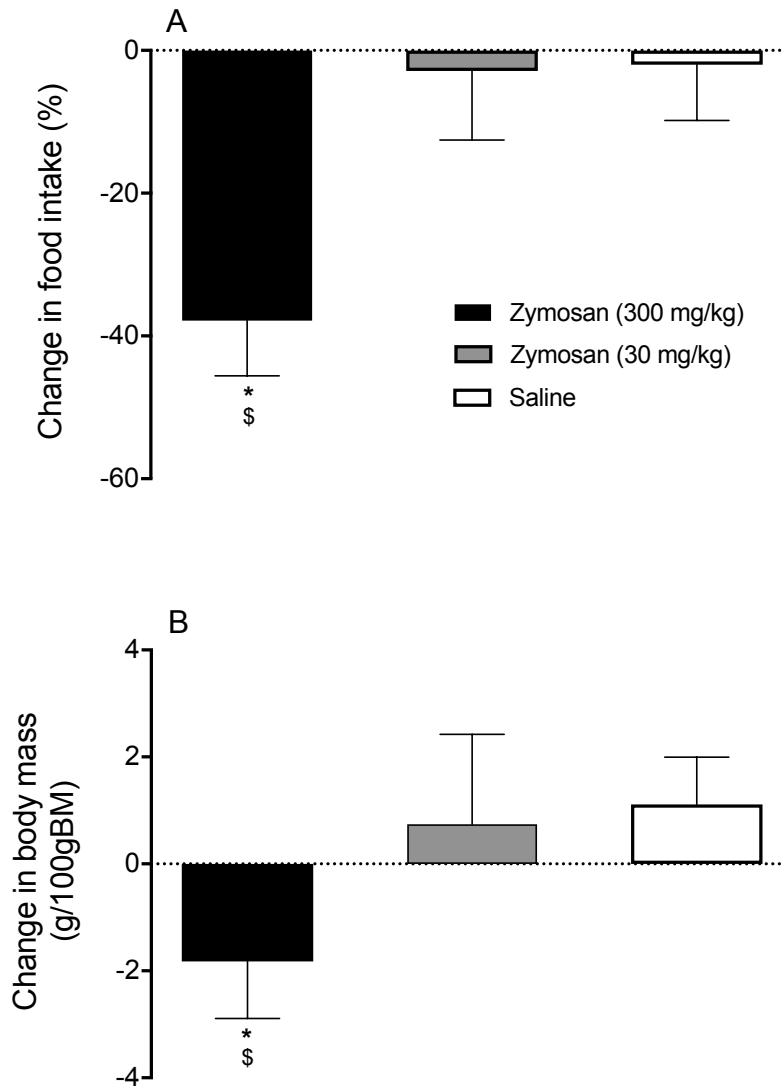


Figure 4.3: Change in food intake (**A**) and body mass (**B**) (mean and SD) of rats 24 hours after subcutaneous injection of either 300 mg/kg zymosan (n = 8), 30 mg/kg zymosan (n = 7) or saline (n = 7). * indicates differences between 300 mg/kg zymosan and saline ($P < 0.001$, Bonferroni), \$ indicates differences between 300 mg/kg and 30 mg/kg zymosan ($P < 0.01$, Bonferroni). A one sample t test indicated that food intake decreased significantly from pre-injection values in rats receiving 300 mg/kg zymosan ($t = 13.9$ and $P < 0.0001$), but not in rats receiving 30 mg/kg zymosan ($t = 0.8$ and $P = 0.46$) or saline ($t = 0.7$ and $P = 0.52$). A one sample t test indicated a significant loss of body mass from pre-injection values in rats receiving 300 mg/kg zymosan ($t = 4.8$ and $P = 0.0019$), but not in rats receiving 30 mg/kg zymosan ($t = 1.2$ and $P = 0.287$); rats receiving saline significantly gained body mass ($t = 3.3$ and $P = 0.0156$).

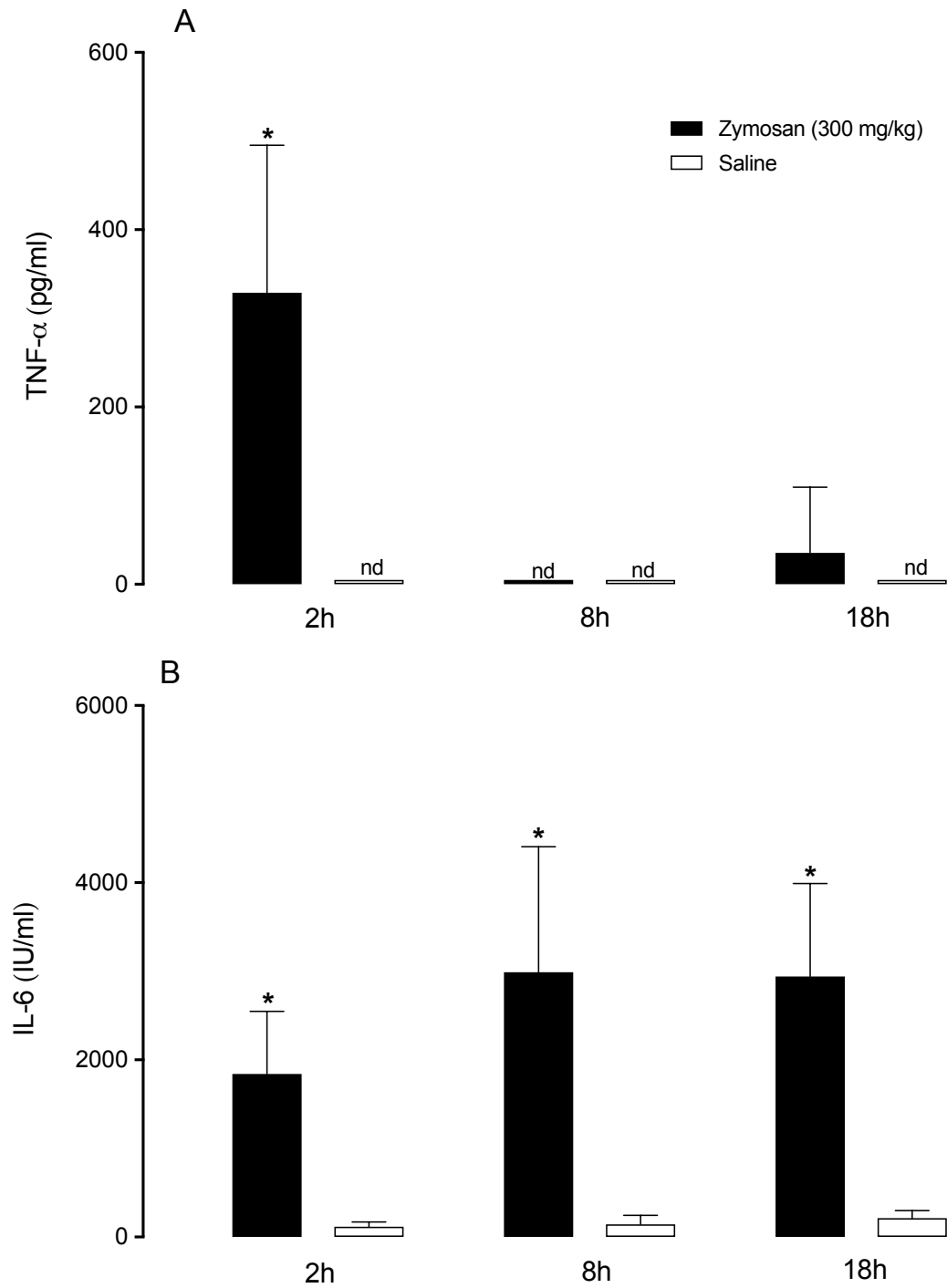


Figure 4.4: Blood plasma levels of bioactive TNF- α (A) and IL-6 (B) at 2, 8 or 18 hours after subcutaneous injection of either 300 mg/kg zymosan or saline. Sample size per intervention group per time point = 4 – 6 rats. * Indicates significant differences between 300 mg/kg zymosan and saline ($P < 0.0001$, Bonferroni); nd = not detectable.

4.3.2.2 *Activation of NF-IL6, STAT3 and COX-2 in the brain*

Figure 4.5 shows representative photomicrographs of immunohistochemical detection of NF-IL6 in the OVLT of rats injected s.c. with 300 mg/kg zymosan or saline. NF-IL6 immunoreactivity, which colocalised with the DNA marker DAPI, appeared in this sensory circumventricular organ as early as 2 hours after injection (Figure 4.5B), was strongest at 8 hours (Figure 4.5C) and was still significantly detectable 18 hours after rats had been injected with zymosan (Figure 4.5D). Rats injected with saline did not show nuclear NF-IL6 immunoreactivity in the OVLT at any of the three time points (Figure 4.5A).

Figure 4.6 shows representative photomicrographs of immunohistochemical detection of STAT3 activation in the OVLT of rats injected with the 300 mg/kg zymosan or saline. The strongest STAT3 immunoreactivity, which colocalised with the nucleic acid marker DAPI, appeared in this sensory circumventricular organ between 2 hours and 18 hours after rats were injected with zymosan (Figure 4.6B - D). Rats injected with saline did not show nuclear STAT3 immunoreactivity at any of the three time points (Figure 4.6A). The nuclear STAT3 signals were closely associated with blood vessels as shown by an additional colocalisation with vWF.

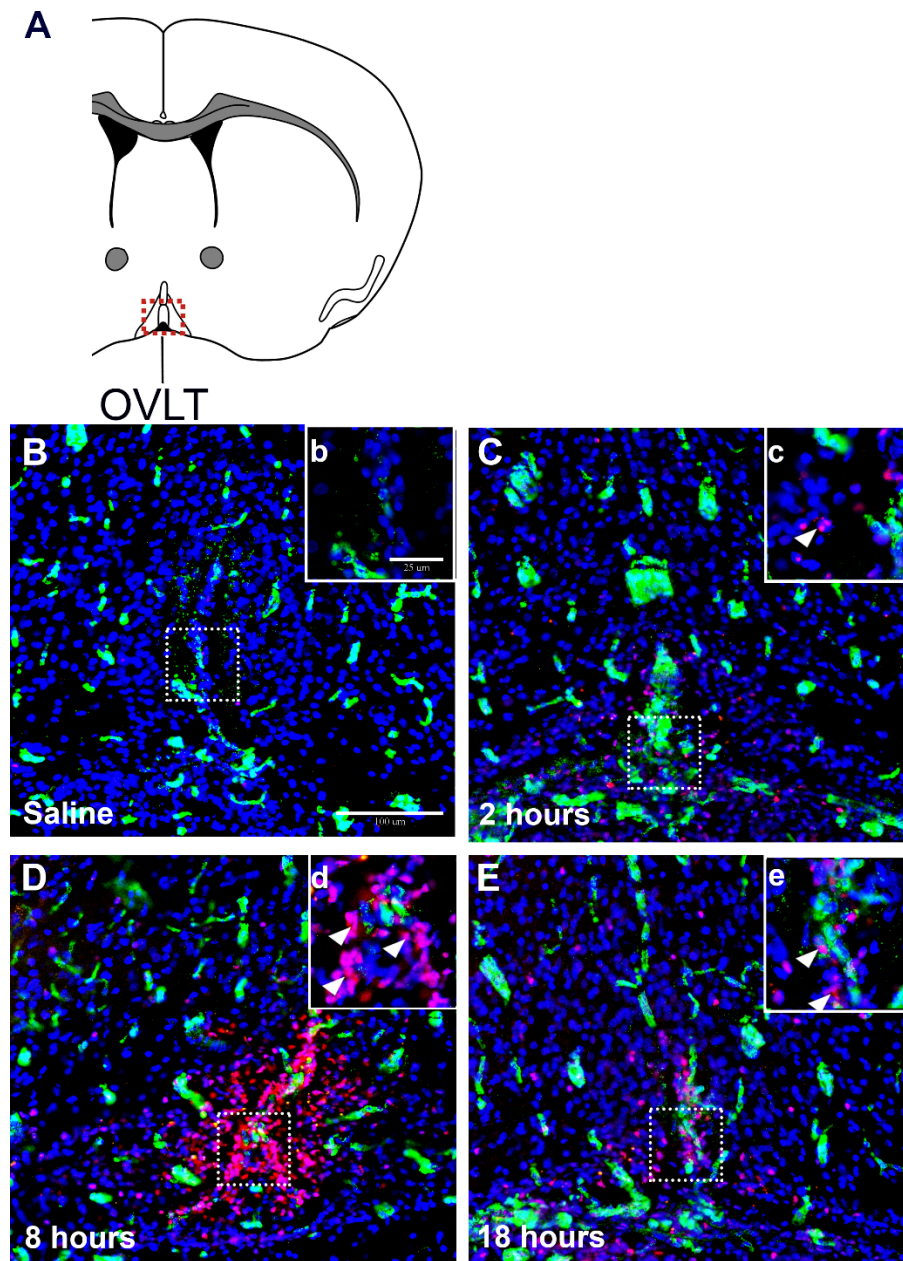


Figure 4.5: Nuclear NF-IL6 immunoreactivity in the OVLT at 2, 8 and 18 hours after subcutaneous injection of either 300 mg/kg zymosan or saline. (A) Schematic overview of the rat brain showing the position of the OVLT. The co-localisation of the red NF-IL6 signals with the nuclear DAPI stain (blue) indicates the nuclear localization of the signals, which appeared at 2, 8 and 18 hours (C - E). Blood vessels were stained with von Willebrand factor (green). NF-IL6 staining (red) was associated with blood vessels as shown by co-localization with von Willebrand factor (green) (white arrowheads in inserts c – e). Subcutaneous injection of rats with saline was not associated with nuclear NF-IL6 immunoreactivity (B). The locations of areas used for high magnification are indicated by a dashed square in the overviews. Blood vessels were stained with von Willebrand factor (green). Scale bar in (B) represents 100 μ m and applies to 20 x magnification micrographs (B - E). Scale bar in (b) represents 25 μ m and applies to 40 x magnification inserts (b - e).

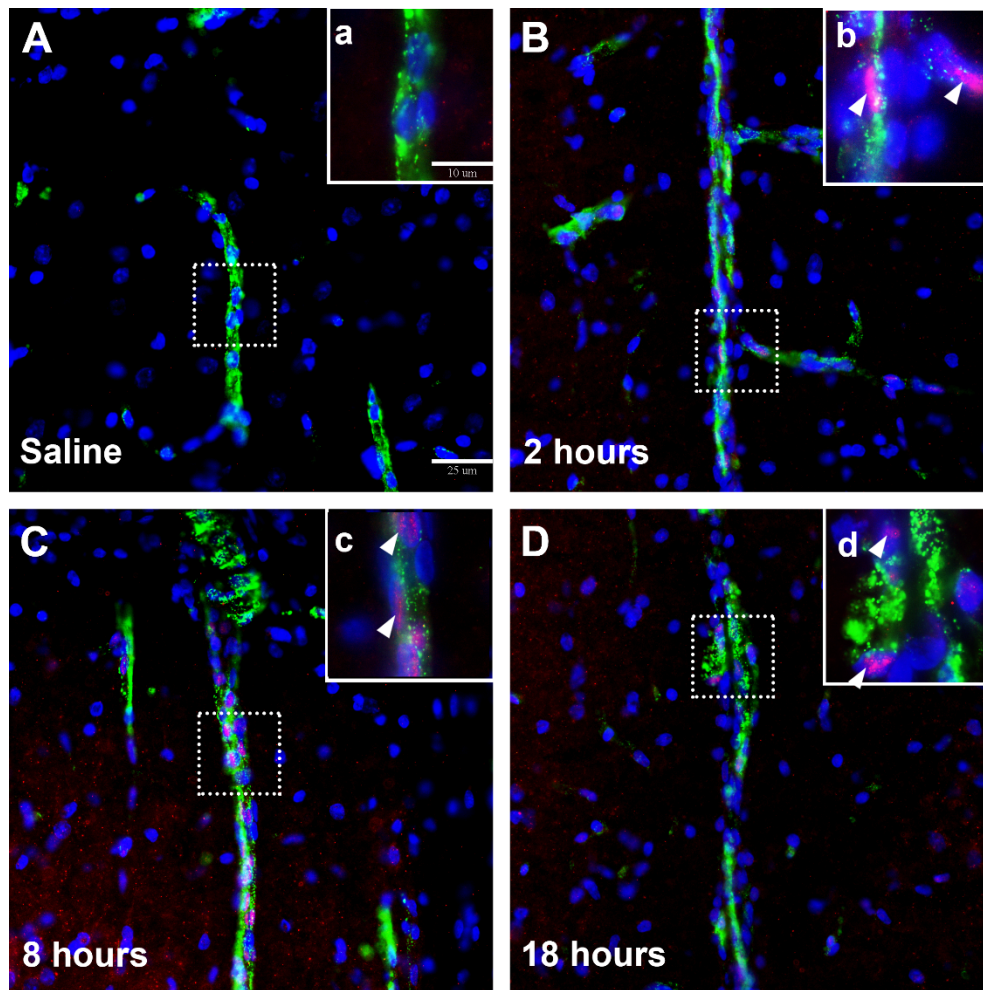


Figure 4.6: Nuclear STAT-3 immunoreactivity in the OVLT at 2, 8 and 18 hours after subcutaneous injection of either 300 mg/kg zymosan or saline. The co-localisation of the red STAT-3 signals with the nuclear DAPI stain (blue) indicates the nuclear localization of the signals, which appeared at 2, 8 and 18 hours (**B - D**). Blood vessels were stained with von Willebrand factor (green). STAT-3 staining (red) was associated with blood vessels as shown by co-localization with von Willebrand factor (green) (white arrowheads in inserts **b - d**). Subcutaneous injection of rats with saline was not associated with nuclear translocation of STAT-3 (**A**). The locations of areas used for high magnification are indicated by a dashed square in the overviews. Scale bar in (**A**) = 25 μ m and applies to all micrographs (**A - D**) which were taken at 40 x magnification. Scale bar in (**a**) = 10 μ m and applies to all inserts (**a - d**) which were taken at 100 x magnification.

Figure 4.7 shows representative high magnification photomicrographs of immunohistochemical detection of the enzyme COX-2 in the OVLT of rats injected with 300 mg/kg. Typical perinuclear COX-2 immunoreactivity was detected in this sensory circumventricular organ between 2 hours and 18 hours after rats were injected with zymosan (Figure 7B - D). The perinuclear COX-2 signals colocalised with blood vessels as shown by an additional colocalisation with vWF (Figure 4.7B and D).

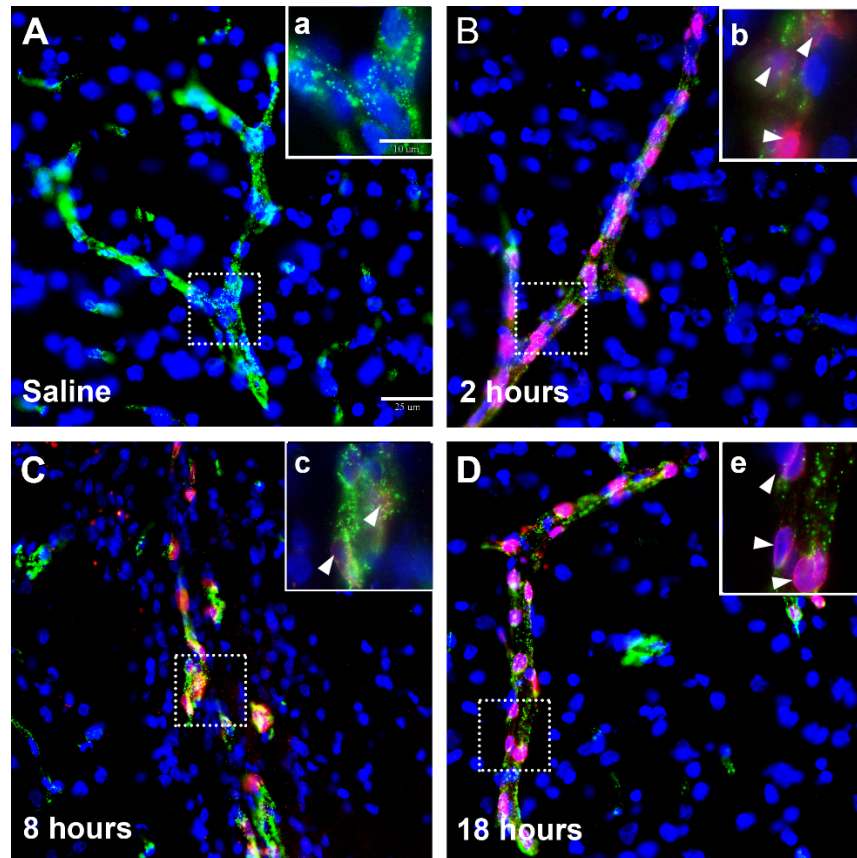


Figure 4.7: Perinuclear COX-2 immunoreactivity at 2, 8 and 18 hours after subcutaneous injection of either 300 mg/kg zymosan or saline. The co-localisation of the red COX-2 signals with the nuclear DAPI stain (blue) indicates the perinuclear localization of the signals, which appeared at 2, 8 and 18 hours (**B - D**). Blood vessels were stained with von Willebrand factor (green). COX-2 staining (red) was associated with blood vessels as shown by co-localization with von Willebrand factor (green) (white arrowheads in inserts **b – d**). Subcutaneous injection of rats with saline was not associated with COX-2 immunoreactivity (**A**). The locations of areas used for high magnification are indicated by a dashed square in the overviews. Scale bar in (**A**) = 25 μ m and applies to all micrographs (**A - D**) which were taken at 40 x magnification. Scale bar in (**a**) = 10 μ m and applies to all inserts (**a - d**) which were taken at 100 x magnification.

Perinuclear COX-2 immunoreactivity was also detected in the walls of large vessels with large lumens in the subarachnoid space, lateral to the optic chiasm (images not shown). No perinuclear COX-2 signals were observed in blood vessels in the OVLT at any of the three time points in rats injected with saline (Figure 4.7A).

4.3.2.3 *Relative mRNA expression of inflammatory mediators in the hypothalamus*

Figure 4.8 shows the increase in hypothalamic-mRNA expression of pro-inflammatory target genes and regulatory proteins of transcription factors; following injection of 300 mg/kg zymosan or saline in rats. Figure 8A and 8B show that rats injected with zymosan had significantly greater mRNA expression of NF-IL6 and I κ B α (the negative regulator of NF- κ B) in the hypothalamus at 8 and 18 hours compared to rats injected with saline (NF-IL6: main effects of time ($F_{(2, 23)} = 3.54$, $P = 0.0458$), treatment ($F_{(1, 23)} = 27.18$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 5.08$, $P = 0.0149$); I κ B α : main effects of time ($F_{(2, 23)} = 2.76$, $P = 0.0843$), treatment ($F_{(1, 23)} = 17.12$, $P = 0.0004$) and interaction ($F_{(2, 23)} = 0.6661$, $P = 0.5233$)).

Figure 4.8C shows that there was a significant increase in SOCS3 mRNA expression in the hypothalamus of rats injected s.c. with zymosan (300 mg/kg) compared to rats injected with saline (main effects of time ($F_{(2, 23)} = 4.27$, $P < 0.0264$), treatment ($F_{(1, 23)} = 147.20$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 4.422$, $P = 0.0237$)). Rats injected with zymosan had significantly higher hypothalamic SOCS3 mRNA expression at 2, 8 and 18 hours compared to rats injected with the saline ($P < 0.01$, Bonferroni).

Figure 8D, 8E and 8F shows that rats injected with zymosan had significantly greater hypothalamic TNF- α mRNA expression at 8 and 18 hours, IL-6 mRNA expression at 8 hours and IL-1 β mRNA expression at 2, 8 and 18 hours compared to rats injected with the saline (TNF- α : main effects of time ($F_{(2, 23)} = 12.47$, $P = 0.0002$), treatment ($F_{(1, 23)} = 31.38$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 5.56$, $P = 0.0107$); IL-6: main effects of time ($F_{(2, 23)} = 0.69$, $P = 0.5113$), treatment ($F_{(1, 23)} = 8.18$, $P = 0.0089$) and interaction ($F_{(2, 23)} = 1.34$, $P = 0.2807$); IL-1 β : main effects of time ($F_{(2, 23)} = 21.68$, $P < 0.0001$), treatment ($F_{(1, 23)} = 153.70$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 15.39$, $P < 0.0001$)).

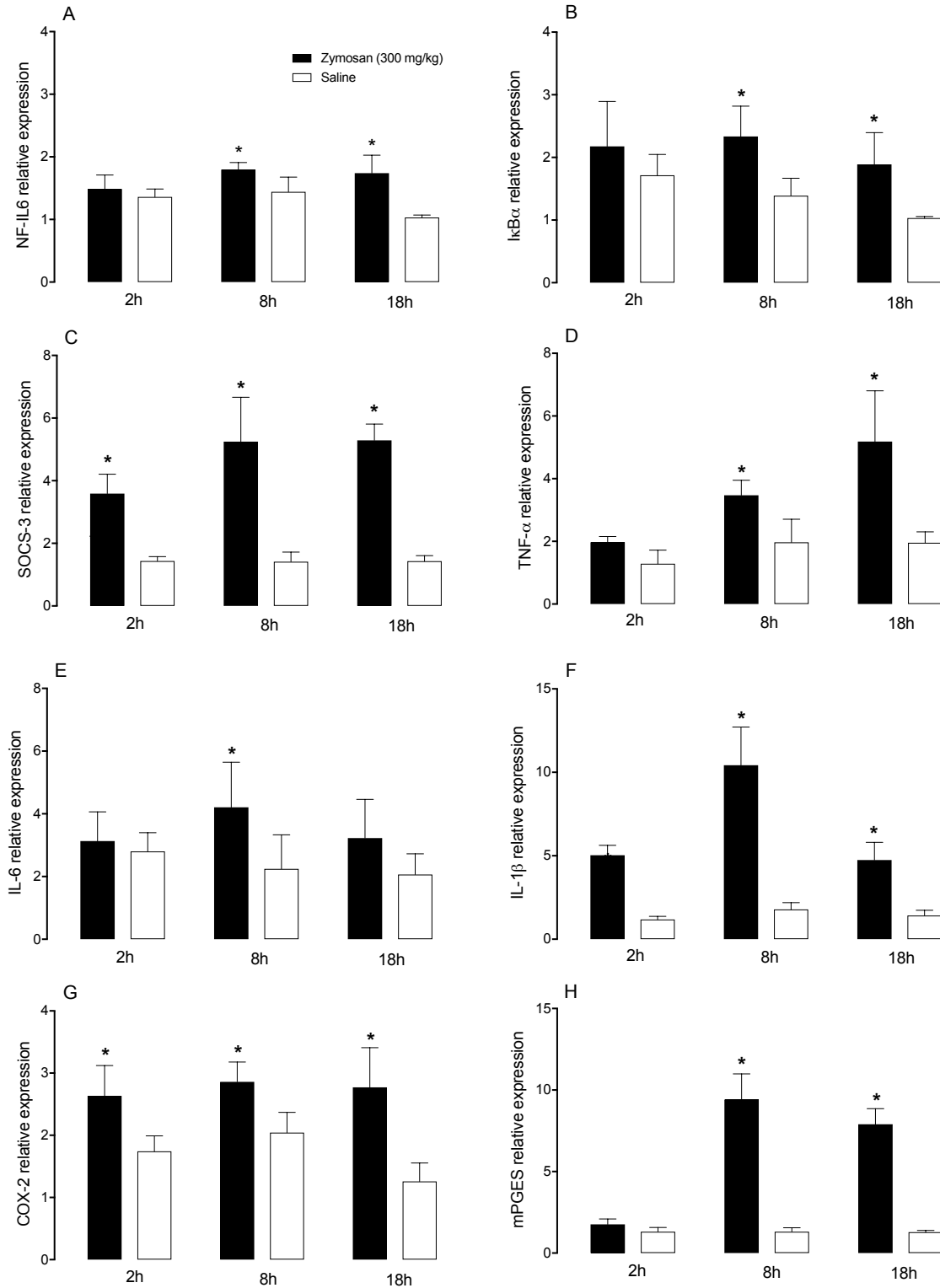


Figure 4.8: Relative mRNA expression (mean and SD) of transcription factors (A - C), pro-inflammatory cytokines (D - F) and rate-limiting enzymes for PGE₂ synthesis (G - H) in the rat hypothalamus at 2, 8 and 18 hours after subcutaneous injection of either 300 mg/kg zymosan or saline. Sample size per intervention group per time point = 4 – 6 rats. * Indicates significant differences between 300 mg/kg zymosan and saline (P < 0.05, Bonferroni).

Figure 4.8G shows that there was an increase in COX-2 mRNA expression in the hypothalamus of rats injected s.c. with zymosan (300 mg/kg) compared to rats injected with saline (main effects of time ($F_{(2, 23)} = 2.33$, $P = 0.1196$), treatment ($F_{(1, 23)} = 42.29$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 1.80$, $P = 0.1879$)). Rats injected with zymosan had significantly higher hypothalamic COX-2 mRNA expression at 2, 8 and 18 hours compared to rats injected with the saline ($P < 0.05$, Bonferroni).

Figure 4.8H shows that there was a significant increase in mPGES mRNA expression in the hypothalamus of rats injected s.c. with zymosan (300 mg/kg) compared to rats injected with saline (main effects of time ($F_{(2, 23)} = 57.04$, $P < 0.0001$), treatment ($F_{(1, 23)} = 265.50$, $P < 0.0001$) and interaction ($F_{(2, 23)} = 57.22$, $P < 0.0001$)). Rats injected with zymosan had significantly higher hypothalamic mPGES mRNA expression at 8 and 18 hours compared to rats injected with the saline ($P < 0.0001$, Bonferroni).

4.4 Discussion

The results obtained in this study show that subcutaneous administration of zymosan in rats induces a dose-dependent sickness response which includes fever, lethargy, anorexia and growth stunting (Figure 4.1 – 4.3). The fever and sickness behaviour are accompanied by an increase in circulating levels of IL-6 and TNF- α (Figure 4.4) and subsequent activation of pro-inflammatory transcription factors (NF-IL6 and STAT-3) in the OVLT between 2 and 18 hours (Figure 4.5 – 4.6). Zymosan administration also induced an increase in hypothalamic expression of genes encoding pro-inflammatory cytokines (IL-1 β , TNF- α and IL-6), regulators of inflammatory transcription factors (IkB α and SOCS3) and rate-limiting enzymes for prostaglandin synthesis (COX-2 and mPGES) (Figure 4.7 – 4.8).

The characteristics of the thermal response to zymosan administration are dependent on the dose of zymosan injected. In this study, s.c. injection of 300 mg/kg of zymosan induced fever with an earlier onset and greater magnitude (time to onset ~2 hours; time to peak = 9 hours and an amplitude of 2 at 18 hours after injection) compared to s.c. injection of 30 mg/kg. My results show that s.c. injection of a moderately high dose (300 mg/kg) of zymosan could be used experimentally for inducing, non-septic and non-hypothermic thermal responses characterised by reasonably long fevers that allow for pharmacological manipulations and antipyretic drug

testing without inducing prolonged suffering and sickness in the rats. In the first 24 hours after injection, the febrile response induced by administration of zymosan was comparable to that induced by 2-4 g/kg Brewer's yeast (see Figure 3.1). As was observed in Brewer's yeast induced fever (Figure 3.1), 18 hours after injection corresponds with the plateau phase of the fever when temperatures are consistently 1.5 - 2 °C above that of normothermic rats. Thus, for zymosan-induced fever at a dose of 300 mg/kg, the 18-hour time point is also an appropriate time point for injecting antipyretics and determining their efficacy in attenuating fever.

In addition to fever, zymosan dose-dependently induced lethargy (Figure 4.2), anorexia and growth-stunting (Figure 4.3). The reduction in activity (~60%) and food intake (~40%) induced 24 hours after zymosan administration is comparable to that induced by the medium and low doses of Brewer's yeast (see Figures 3.2 - 3.3). Sickness behaviour was prominent during the night period, when the rats are usually active and consume most of their food. Thus, if one intends to measure the efficacy of a drug against zymosan-induced sickness behaviour, they may consider injecting the pharmacological drug before the night phase. The observation that zymosan administration is associated with sickness behaviour is consistent with the findings of other researchers who also reported anorexia (Cremeans-Smith and Newberry, 2003, Fantuzzi et al., 1997), lethargy (Hubschle et al., 2007) and weight loss (Hubschle et al., 2007, Volman et al., 2005) during zymosan-induced inflammation.

The fever, lethargy, anorexia and body mass stunting induced by s.c. administration of 300 mg/kg zymosan was accompanied by an increase in the blood plasma levels and hypothalamic mRNA expression of the pro-inflammatory cytokines IL-6 and TNF- α (Figure 4.4 and 4.8). Circulating levels of TNF- α were significantly increased 2 hours after zymosan administration suggesting that TNF- α release, which precedes fever, may mediate the early rise in temperature during zymosan-induced fever. Early TNF- α release during zymosan-induced inflammation has similarly been reported by other researchers (Asmuth et al., 1990b, Fantuzzi et al., 1997, Utsunomiya et al., 1998) and inhibition of TNF- α activity blocked the earlier part of zymosan-induced fever as opposed to the latter (Bastos-Pereira et al., 2014, Kanashiro et al., 2015).

I also observed elevated plasma levels of IL-6 during the early phase (2 hours), as well during the mid-to-late plateau phase (8 and 18 hours) after zymosan injection. The timing of IL-6 increase suggests that IL-6 may be involved in both early development and the maintenance of

zymosan-induced fever, as has similarly been noted in Brewer's yeast-induced fever (see Figure 3.4). An early (3 hours) increase in IL-6 has been reported in rats injected intraperitoneally with zymosan (Bastos-Pereira et al., 2014). Additionally, inhibition of IL-6 activity attenuated zymosan-induced fever (Bastos-Pereira et al., 2014, Kanashiro et al., 2015). These data, together with my current findings, support the involvement of IL-6 in mediating zymosan-induced fever.

Zymosan-induced fever and sickness behaviour are mediated by prostaglandins (Lima et al., 2016) and zymosan has been shown to activate the production of cytokines (TNF- α and IL-6) by neuroglial cells cultured from the OVLT and MnPO (Bastos-Pereira et al., 2014). As discussed in **Chapter 1** and **3**, production of PGE₂ and central cytokines occurs via activation of inflammatory transcription factors. To my knowledge, my results provide the first, *in vivo*, experimental evidence that zymosan administration induces a time-dependent activation of the pro-inflammatory transcription factors NF-IL6 and STAT3 in the OVLT and their target genes in the hypothalamus (Figure 4.5 - 4.8). These findings align with the current theories describing the development of fever in which humoral immune signals are believed to activate the brain to synthesize inflammatory molecules that then act locally to induce fever and sickness behaviour (Rummel, 2016, Saper and Breder, 1994).

The activation and nuclear translocation of NF-IL6 in the OVLT observed in this study occurred in the early and the mid-to-late plateau phases of zymosan-induced fever (Figure 4.5). Additionally, the strongest activation of NF-IL6 in the OVLT occurred just before the highest temperatures were achieved; 8 hours after injection (see Figure 4.5 and Figure 4.1). The late induction of NF-IL6 (strongest immunoreactivity at 8 hours) is similar to the induction pattern reported for Brewer's yeast (see Figure 3.5) and supports the hypothesis that NF-IL6 may be more active during the latter, than earlier phase of the zymosan induced-acute phase response. Overall, the activation of NF-IL6 in the OVLT by zymosan carries an important functional significance because, as discussed in **Chapter 1**, this sensory CVO lies close to and has numerous neural connections with the febrigenic zones of the hypothalamus. The OVLT may therefore be an important port of entry for immune signals originating in the periphery in zymosan-induced sickness responses.

Some researchers have argued that there is transcriptional synergism between NF-IL6 and NF- κ B and that the two transcription factors co-regulate gene expression (Matsusaka et al., 1993,

Santos et al., 2011). Previous studies have also shown that zymosan activates the NF- κ B pathway by binding to TLR2/TLR6 and Dectin 1 (de Hooge et al., 2004b). Although I did not perform immunohistochemical staining of NF- κ B in this study, I measured the expression of its regulatory protein, I κ B α , whose upregulation is an established and accepted indicator of NF- κ B activation (Quan et al., 1997). I noted a late expression of hypothalamic I κ B α (8 and 18 hours), which confirms earlier reports given from cell culture studies that the zymosan-induced inflammatory response may be mediated by both the NF-IL6 and NF- κ B pathways (Santos et al., 2011).

In addition to evidence showing the activation of NF-IL6 and NF- κ B, I also observed an early activation of STAT3 associated with blood vessels in the brains of rats injected with zymosan (Figure 3.6). These findings suggest that in addition to stimulating NF- κ B and NF-IL6 signalling pathways, zymosan may also induce neuroinflammation through activation of the JAK/STAT signalling pathway. The involvement of STAT3 in the zymosan-induced sickness response could be further confirmed by the increase in the expression of the STAT3 regulator (SOCS3) observed in the hypothalamus.

The activation and nuclear translocation of NF-IL6 and STAT3 in the brain induces the transcription of inflammatory genes including cytokines and rate-limiting enzymes for PGE₂ synthesis (COX-2 and mPGES) (Damm et al., 2012, Matsusaka et al., 1993, Schneiders et al., 2015, Rummel et al., 2011). NF-IL6 and STAT3 have been shown to play a role in the regulation of transcription of genes encoding the pro-inflammatory cytokines, IL-6 and IL-1 β (Zhang et al., 1994, Zhang and Rom, 1993, Rummel et al., 2011). The transcriptional regulation of these genes in the hypothalamus following zymosan administration is, for the first time, shown in this study. I observed an upregulation in the expression of genes encoding TNF- α , IL-6 and IL-1 β in the hypothalamus. Given the numerous physiological functions of the hypothalamus i.e. control of thermoregulation, feeding, drinking, sleep, an activation of inflammatory mediators in the hypothalamus is of functional significance as it may explain the behavioural shift that occurs during the zymosan-induced sickness response.

Zymosan-induced sickness is ultimately mediated by prostaglandins as has been shown in studies where; (1) systemic injection of zymosan was associated with an increase in PGE₂, (2) stimulation of macrophages with zymosan induced the production of PGD₂, PGE₂, PGF₂ and

PGI₂, and (3) the administration of prostaglandin-synthesis inhibitors such as indomethacin blocked the sickness response induced by zymosan injection (Lima et al., 2016). Prostaglandins, particularly PGE₂, are produced in the brain and act as the terminal mediators of infection-induced neuroinflammation (Ivanov and Romanovsky, 2004). The activation of COX-2 in cerebrovascular cells (see Figure 4.7) and the upregulation of COX-2 and mPGES genes in the hypothalamus of rats injected with zymosan provide further evidence to confirm the involvement of PGE₂ in mediating zymosan-induced sickness.

Overall, the mechanisms underlying the zymosan-induced sickness response are similar to those mediating Brewer's yeast-induced sickness. Zymosan, like Brewer's yeast, induces the production of the peripheral cytokines TNF- α and IL-6. At the doses given in this study, the degree of increase of the pro-inflammatory cytokines was much lesser when zymosan was administered than when yeast was administered (see Figures 3.4 and 4.4). Except for IL-1 β and COX-2, the degree of increase in the expression of hypothalamic genes (NF-IL6, IkB α , SOCS3, TNF- α , IL-6 and mPGES) was much lower when zymosan was administered than when yeast was administered (see Figures 3.8 and 4.8). A typical example is that of mPGES whose expression increased almost 40-fold in Brewer's yeast-induced sickness but only about 10-fold in zymosan-induced sickness. These results confirm the potency of the dose of live yeast that is currently being administered during antipyretic screening while affirming the suitability of using zymosan as an alternative pyrogen to induce a full suite of inflammatory mediators at a moderate degree.

In conclusion, the results obtained from this study show that s.c. administration of zymosan induces a reasonably long-lasting fever that is accompanied by sickness behaviour. Administration of zymosan, like Brewer's yeast, is associated with an increase in the levels of peripheral cytokines (IL-6 and TNF- α), activation of inflammatory transcription factors (NF-IL6, NF- κ B and STAT3), and an upregulation of their target genes (central cytokines and inducible enzymes) in the hypothalamus. The ability of zymosan to induce peripheral and central inflammatory mediators supports its suitability as an experimental pyrogen. In zymosan-induced sickness, just as in Brewer's yeast-induced sickness, the 18-hour time point is more appropriate for assessing the efficacy of a pharmacological agent against fever while both the 8-hour and 18-hour time points appear suitable for determination of mechanisms of action.

4.5 References

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CHAPTER FIVE - CONCLUSIONS

Since the pre-antibiotic era, and even now after the discovery of antibiotics, antipyretic drugs remain useful for reducing elevated body temperatures associated with infectious diseases such as malaria and influenza, and even in non-infectious, autoimmune and auto-inflammatory disorders. Fever control using antipyretic drugs has also proven important in vulnerable populations such as children with the risk of developing febrile seizures, critically ill patients and in patients with cardiovascular complications. The most commonly used assay for detecting antipyretic potential of drugs has not changed much over the past century. The recommended antipyretic drug testing assay entails inducing fever in rats by subcutaneous injection of 2 – 4 g/kg of Brewer's yeast solution. A test drug is then injected 18 hours after the injection of the pyrogen and thereafter rectal temperatures are measured at hourly intervals, for five hours to determine the efficacy of the test drug in attenuating fever. Surprisingly, although this recommended protocol is extensively accepted and commonly used for testing antipyretics particularly in pharmacognostic studies, there is no clear scientific evidence provided to support the pyrogen used for inducing fever and the method used for measuring temperature.

The general aim of my thesis was to validate specific aspects of the currently recommended anti-pyretic testing protocol and to provide experimental evidence to support the basis of its continued use. I also sought to establish avenues to improve the current published protocol to ensure accuracy and reliability of results (Thangaraj, 2016, Vogel, 2002). More specifically, my first objective was to examine the method commonly used for measuring temperature during antipyretic screening and in this study, I investigated the effects of rectal temperature measurement on the thermal profiles of normothermic and febrile rats (**Chapter 2**). I further compared temperatures recorded using rectal thermocouple probes with temperatures measured using intra-abdominally implanted radiotelemeters. My second objective was to characterise the fever, sickness behaviour and inflammatory response induced by Brewer's yeast, the pyrogen that is recommended for inducing fever during antipyretic screening (**Chapter 3**). Having characterised the sickness response to Brewer's yeast and having established the limitations associated with injecting the current recommended doses, my last objective was to investigate if zymosan (a cell wall moiety of Brewer's yeast) could be used as an alternative experimental pyrogen during antipyretic screening (**Chapter 4**). My major findings are discussed below in the context of current literature.

5.1 Protocol for testing antipyretic drugs

5.1.1 *Measuring body temperature*

As discussed in **Chapter 1**, a good thermometry method is one which has a high level of sensitivity and accuracy; one that is associated with minimum distress and measurement-related artefacts. As outlined above, the protocol for testing antipyretic drugs entails the use of a thermocouple probe to measure rectal temperature at hourly intervals after the injection of the test antipyretic drugs. There are a number of reasons that could explain why the use of rectal thermometry is very common in antipyretic testing particularly in ethnopharmacological studies. Firstly, rectal thermometry has relatively low set-up and maintenance costs when compared to other methods such as biotelemetry. Secondly, as shown in **Chapter 2**, core body temperatures measured using rectal thermometry are qualitatively comparable to those measured using biotelemetry. Although I observed a linear association between values measured using the two methods, the agreement interval of the two methods shows that temperatures measured using thermocouple probes can be greater (up to 0.7 °C) or lower (up to 0.5 °C) than those obtained with a radiotransmitter. Additionally, rectal thermometry underestimated the amplitude of the fever at 18 hours by approximately 0.4 °C. Thus, rectal thermometry and biotelemetry may yield qualitatively similar results but rectal thermometry may underestimate or overestimate core body temperatures. The difference between temperatures measured using the two methods is likely caused by differences in the depth of probe insertion. In this study, even though I used and maintained an insertion depth of 40 mm (reportedly used successfully in studies done using the same size of rats as were used in this study), I still observed differences between temperatures measured using rectal thermometry with those recorded using radiotransmitters. The accuracy of rectal thermometry depends not only on the techniques of the researcher, but so too on the cooperation of the animal, which is difficult to standardize.

Although it is a recommended, popular method for measurement of thermal responses in pharmacological studies, my results show that rectal temperature measurement is associated with stress-related increases in temperature. The stress-induced hyperthermia is clear, consistent and reproducible in normothermic rats, but muted in febrile rats. The difference in the thermal responses of normothermic vs febrile rats to stress has

significant implications that researchers in pharmacological studies need to consider. Usually, the antipyretic efficacy of a drug is determined by the index of similarity or difference between febrile rats treated with the test drug and the normothermic untreated controls. Due to the stress-related increase in temperature in normothermic rats, coupled with no change in their febrile counterparts, there is a higher likelihood of statistical non-significance which may be erroneously mistaken to be positive drug effects. However, it is important to note that the experiments in **Chapter 2** were carried out at temperatures below the thermoneutral zone of rats and the thermal responses to stress could have been different had they been done at thermoneutral temperatures.

Some may argue that the stress-induced hyperthermia resolves within one hour and that since common practice in pharmacological studies is to measure rectal temperatures once every hour, then stress-induced hyperthermia is unlikely to affect the accuracy of the results. However, from the literature reviewed in my thesis, I observed that it is also common for researchers to take rectal temperature measurements at even shorter intervals e.g. 30 minutes [for example see (Josa et al., 2001, Liu et al., 2016, Pingsusaen et al., 2015, Zakaria et al., 2006)]. At these shorter intervals, the reported thermal responses reported are likely to reflect the hyperthermia. Additionally, the procedure of rectal temperature measurement is also associated with other physiological changes such as an increase in cytokines and circulating hormones with longer lasting effects that may influence physiological responses long after the hyperthermia has subsided (Veening et al., 2004). Thus, although a rat's temperature may have decreased back to normal after an hour, its biochemical make-up and physiological state may still be different to that of an unstressed rat.

Another equally important observation I made regarding the use of rectal thermometry was that the protocol commonly employed for “habituating” rats to the procedure does not alleviate or blunt the development of stress-induced hyperthermia. One plausible explanation for the lack of adaptation could be that stress-induced hyperthermia is part of the non-adaptive sympathetically driven stress responses. Other researchers have shown little-to-no adaptation in the heart rate and catecholamine levels in response to repeated exposure to psychosocial stress (Schommer et al., 2003). Future studies could possibly investigate effective protocols for habituating rats to the procedure of rectal temperature

measurement and establish if rats can be conditioned to a point where they blunt the stress-related hyperthermia.

In summary, although it potentially yields similar results to biotelemetry, the procedure of rectal temperature measurement is associated with a number of technical challenges which include stress-induced hyperthermia, a lack of clearly defined habituation protocols and difficulties in the standardization of measurements and techniques. These challenges have negative implications on the accuracy of thermal responses reported during antipyretic drug-testing. Thus, when compared with rectal thermometry, surgically-implanted radiotelemeters appear to be a more reliable and better tool for accurate, repeated temperature measurement during antipyretic screening.

5.1.2 Pyrogen and timing of anti-pyretic drug administration

As important as it is to choose a good method for measuring temperature during antipyretic drug testing, it is equally important to consider the efficacy of the pyrogen selected for inducing fever. As previously discussed in **Chapter 1**, the success of an experimental pyrogen lies in its ability to induce reasonably uniform, reproducible fevers that last long enough to allow pharmacological manipulations. My results show that when injected subcutaneously, Brewer's yeast is an immunogenic substance capable of inducing fever in rats. The immunogenicity of Brewer's yeast therefore forms the basis for its long-standing use and recommendation as an experimental pyrogen. However, the dose recommended for inducing sickness during antipyretic testing (4g/kg) induces an initial short-lived hypothermia followed by a long-lasting fever. What is clear from my findings is that the hypothermia was reproducibly associated with injection of the high but not lower doses of Brewer's yeast suggesting that it may be related to hyperactivation of the immune response as occurs during sepsis. Current guidelines for antipyretic screening do not specify the environmental temperatures at which the experiments should be conducted. However, previous studies have shown that the thermal response of rodents to a pyrogen varies with environmental temperatures. When injected under their thermoneutral zone, rats have been shown to decrease temperature prior to developing a fever (Rudaya et al., 2005). It is possible, therefore, that the short-lived hypothermia that developed in rats injected with the high dose of Brewer's yeast could be the result of the

ambient temperature at which they were kept, which was below their thermoneutral zone. Future studies characterising the thermal response to Brewer's yeast administration could explore if the same thermal profile observed in the findings in **Chapter 3** would be observed if experiments were to be done at thermoneutral temperatures for rats.

The basis for injecting the test drug at the 18-hour time point is not well explained in literature, but my findings in **Chapters 2 and 3** show that the 18-hour time point falls within the plateau phase of Brewer's yeast-induced fever. Provided the pyrogen is injected just before the dark phase of the light/dark cycle, temperatures will be consistently higher than that of normothermic rats at 18 hours after Brewer's yeast administration. However, had the pyrogen been injected in the early part of the light phase cycle (morning), the 18-hour time point would occur during the night period when the temperatures of controls were naturally high due to the circadian rhythm of core temperature and thus the fever amplitudes would have been smaller. While fever amplitudes may better be monitored during the light phase, sickness symptoms such as lethargy and anorexia are best measured during the dark phase of the circadian cycle; when the rats are active and eating. Although at present they are not included in the current guidelines for antipyretic testing, these sickness symptoms may be useful measurable indices of sickness that can be incorporated in comprehensively testing the efficacy of drugs against the sickness response.

Although the 18-hour time point appears to be a good time point for comparison of temperatures, my results show that inflammatory mediators were more prominent at 8 hours. Thus researchers investigating the mechanism of action of pharmacological substances, could consider administering test drugs before or at 8 hours after administration of Brewer's yeast. In this study the levels of inflammatory mediators were not monitored beyond 72 hours and therefore mediators responsible for maintenance of the fever and sickness behaviours beyond this time point remain to be elucidated. Additionally, other inflammatory markers, not investigated in this study could also be involved in mediating Brewer's yeast induced fever and may also be important in testing the mechanisms of antipyretic drugs. Examples of other inflammatory mediators that have been implicated in mediating LPS-induced fever but their role in yeast-induced fever is still unexplored include leptin, COX-1/COX-3, neuropeptides and chemotactic

cytokines such as macrophage inflammatory proteins (Sachot et al., 2004, Harden et al., 2006, Miñano et al., 1996).

The welfare of the experimental animals is an important factor that may be overlooked by researchers when they choose protocols for antipyretic drug-testing. While Brewer's yeast is cheap and has the immunogenic properties that qualify it as an experimental pyrogen, the high doses of live yeast required to induce fever often cause suffering and discomfort to the experimental animals. Firstly, the recommended high dose of Brewer's yeast (4 g/kg) is injected as a large volume (20 ml/kg) slurry solution that requires large gauge (18G) hypodermic needles to inject and often requires two injection sites. Although the injection sites were on the nape of the neck, injection of the yeast was often accompanied by severe inflammation and swelling in the whole head region on the first day after injection. Secondly, after injection the yeast solution is encapsulated forming an abscess, which although probably aimed at preventing the spread of the pathogen, may have acted as a reservoir for slow release of PAMPs leading to the excessively prolonged sickness response. I also observed however that injecting rats with the medium dose induced a similarly excessive sickness response, the duration and magnitude of which was not much less than that induced by the high dose. The use of the medium dose of Brewer's yeast therefore did not improve the protocol as far as animal welfare was concerned. The lowest dose used induced a very short-lived sickness response that was too short to allow for pharmacological testing. Owing to the animal welfare challenges associated with the excessive sickness induced by the high and medium doses of Brewer's yeast I decided to investigate the possibility of using zymosan as an experimental pyrogen and to examine if this moiety of the yeast cell wall would induce the same suite of inflammatory mediators as does whole yeast.

Subcutaneous administration of a moderately high (300 mg/kg) dose of zymosan induced reasonable fever and sickness behaviour that lasted long enough to allow for pharmacological intervention without inducing unnecessary suffering to the experimental animals. Zymosan administration was also associated with an increase in the same suite of inflammatory mediators in the periphery and the brain as those noted during Brewer's yeast-induced sickness. Thus, current guidelines for antipyretic testing could consider incorporating zymosan as a pyrogen that can be used for inducing fever and sickness behaviours during antipyretic screening. In addition to inducing fever and sickness

behaviour, recent studies have also shown that zymosan-induced fever can be reduced by currently known antipyretics e.g. indomethacin and celecoxib (Bastos-Perreira et al., 2017). The mechanisms underlying zymosan-induced fever therefore appear to be the same mediating most common febrile responses hence making it a suitable pyrogen for testing antipyretics.

The current pharmacognostic protocol for antipyretic testing is based on a model of fungal infections and therefore may not provide a complete picture of the efficacy of test drugs against bacterial and viral infections. Because my study focused only on the most commonly recommended models for antipyretic drug testing, my characterisation was limited to Brewer's yeast and its moiety zymosan and therefore did not include other bacterial or viral pyrogens. In Table 5.1, I have drawn a general comparison of the inflammatory response observed by other researchers during systemic and localised inflammation induced by LPS in rodents with what I observed in Brewer's yeast and zymosan-induced sickness. Fever and sickness behaviours induced by the viral mimetic Poly I:C has also been shown to be mediated by the same inflammatory mediators and downstream pathways as LPS, Brewer's yeast and zymosan (Field et al., 2010, Fortier et al., 2004). These pyrogens appear to share common downstream mediators but the spatiotemporal appearance of these mediators may vary due to the activation of different upstream pathogen recognition receptors. For example, fungal pathogens interact with TLR 2, 6 and dectins, while bacteria and viruses interact with TLR4 and TLR3 respectively. Current guidelines for testing antipyretics could incorporate other pyrogens of bacterial and viral origin e.g. LPS and Poly (I:C) respectively to complement Brewer's yeast or zymosan in the determination of the efficacy of antipyretic drugs.

In **Chapter 3 and Chapter 4**, I provide the measurable indices of sickness, both physiological and behavioural, associated with Brewer's yeast and zymosan administration. These characteristics of the sickness response will help researchers to determine appropriate times for pharmacological interventions while the measurable physiological changes can act as a yardstick for determining the efficacy of test drugs. However, for one to successfully test an antipyretic drug there are a number of other important factors that they will have to consider which include: (1) the appropriate route of administration of the test drug; based on the physicochemical characteristics of the drug, (2) effective doses; based on the quantity and bioavailability of bioactive

compounds (normally requires rigorous chemical analyses) and (3) an appropriate time and frequency of administration which is dependent on the pharmacokinetics of the drug i.e. the rate of absorption, distribution and clearance. The determination of all these were outside the scope of this study's objectives therefore I did not test any antipyretic drugs.

Table 5.1: Comparison of the inflammatory and sickness responses observed in rodents administered yeast, zymosan or LPS

Response	Brewer's yeast (2-4 g/kg; s.c.)	Zymosan (300 mg/kg; s.c.)	Lipopolysaccharide (10 µg – 250 mg/kg; i.p or s.c)	
			References	
Fever	✓	✓	✓	(Harden et al., 2006, Schneiders et al., 2015)
Lethargy	✓	✓	✓	(Harden et al., 2011, Schneiders et al., 2015)
Anorexia and body mass stunting	✓	✓	✓	(Harden et al., 2006, Harden et al., 2011, Layé et al., 2000)
Increased plasma IL-6	✓	✓	✓	(Harden et al., 2011, Miller et al., 1997, Copeland et al., 2005)
Increased plasma TNF-α	✓	✓	✓	(Roth et al., 2000, Copeland et al., 2005)
Increased plasma IL-1β	?	?	✓	(Harden et al., 2011, Layé et al., 2000, Copeland et al., 2005)
NF-κB activation in the OVLT	✓	✓	✓	(Rummel et al., 2006)
STAT-3 activation in the OVLT	✓	✓	✓	(Rummel et al., 2006, Rummel et al., 2004, Harré et al., 2002)
NF-IL6 activation in the OVLT	✓	✓	✓	(Schneiders et al., 2015, Damm et al., 2011)
COX-2 activation in endothelial cells	✓	✓	✓	(Damm et al., 2011, Rummel et al., 2006)
Upregulation of the expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the hypothalamus	✓	✓	✓	(Schneiders et al., 2015, Damm et al., 2011)

Upregulation of the expression of inducible enzymes (COX-2 and mPGES) in the hypothalamus	✓	✓	✓	(Schneiders et al., 2015, Rummel et al., 2006, Damm et al., 2011)
Upregulation of the expression of regulators for transcription factors (SOCS3 and IκBα)	✓	✓	✓	(Schneiders et al., 2015, Rummel et al., 2006)

Based on the findings obtained in this experiments described in this thesis, Table 5.2 summarises the recommendations that could be incorporated into the current protocol in order to improve animal welfare and more importantly improve accuracy and reliability of findings during antipyretic drug-testing.

5.2 Clinical relevance and recommendations

Saccharomyces cerevisiae is a ubiquitous yeast and has for a long time been regarded as safe and non-pathogenic. A growing body of evidence has however implicated *S. cerevisiae* as a causative agent for invasive fungal infections particularly in immunocompromised and postoperative patients suggesting that virulent and pathogenic strains of the yeast may be emerging (Aucott et al., 1990, Cimolai et al., 1987, Clemons et al., 1994, Muñoz et al., 2005). Thus, an understanding of yeast-immune interaction mechanisms and a knowledge of the sickness response associated with yeast infection would be useful in the development of therapeutic targets and management strategies for *S. cerevisiae* infections. My results provide the first experimental evidence of the involvement of some neuroinflammatory signalling pathways and mediators in the *S. cerevisiae*-induced sickness response. The lengthy febrile response and body mass-stunting which are characteristic of Brewer's yeast-induced sickness described in this study, could also serve as useful, measurable clinical indicators during diagnosis and treatment of *S. cerevisiae* infections.

Table 5.2: Recommended modifications to the current protocol for antipyretic testing

	Current recommended protocol	Recommended modifications and rationale	Future work
Animals	Male or female young rats	Male rats- thermal responses in female rats may be affected by the oestrous cycle	Comparison of thermal responses of males and females to consider effects of the oestrous cycle
Pyrogen	2 – 4 g/kg	Use of zymosan as alternative pyrogen since high doses of Brewer's yeast are associated with animal welfare challenges;	Development of protocols that utilise other pyrogens e.g. of bacterial or viral origin during antipyretic testing
Route and site of administration	Subcutaneous; Dorsum of neck	Maintain- good success rate and reproducibility	
Time of injection	Not specified	Injection of Brewer's yeast or zymosan just before dark phase so that the plateau phase (18 hours) falls during day	
Method of temperature measurement	Rectal thermometry	Biotelemetry since rectal thermometry is associated with stress-induced hyperthermia and technical limitations which affect accuracy, reliability and reproducibility or results.	Determination of the effective habituation protocols for rectal thermometry
Time and intervals of temperature measurement	18 hours after pyrogen injection; hourly	Plateau phase – 18 - 23 hours (or beyond) for Brewer's yeast and 9-18 hours for zymosan. Temperatures are consistently high and do not change much during this phase	Assessment of thermal responses in the thermoneutral environment
Measurement of inflammatory mediators	Not included	Measurement of cytokines, transcription factors and hypothalamic gene expression to determine the mechanisms of action of test drugs	Determination of other inflammatory mediators e.g. COX-1, COX-3, leptin

Measurement of sickness behaviours	Not included	Inclusion of sickness behaviours during antipyretic testing because these are a part of the sickness response and are often a cause of discomfort in patients. NB: Cage activity and food intake should be monitored during the dark phase.
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The individual contribution of each cytokine or signalling pathway to the febrile response or sickness behaviours induced by Brewer's yeast was beyond the scope of my thesis and therefore was not thoroughly investigated. The studies I undertook sought to investigate the mediators that are activated during Brewer's yeast-induced sickness, as a starting point in delineating the signalling pathways and mechanisms subserving the Brewer's yeast-induced sickness response. However, the use of cytokine specific antisera and antagonists, if employed in future studies, may shed light on the contribution of each cytokine to the Brewer's yeast-induced sickness response. Additionally, more clinical studies measuring blood cytokines in patients clinically confirmed to have *S. cerevisiae* infections may also assist us to further understand the involvement of these mediators during *S. cerevisiae* infection.

In conclusion, I have provided the experimental evidence and explained the deficiencies of the current protocol used during antipyretic screening in pharmacognostic studies. I have highlighted the shortcomings of using rectal thermometry and have shown instead the reliability and reproducibility offered by biotelemetry. My results also clearly show the lengthy, excessive sickness response associated with injecting the large, recommended doses of Brewer's yeast. To circumvent the animal welfare challenges associated with Brewer's yeast administration, I have demonstrated that zymosan could be a useful alternative pyrogen to induce fever and sickness behaviour. My findings also provide the first experimental evidence of the involvement of inflammatory mediators in the *S. cerevisiae*-induced sickness response, an important first step towards the delineation of the mechanisms underlying *S. cerevisiae* infections.

5.3 References

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APPENDICES

APPENDIX 1: Ethics clearance certificate



STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/10/C

APPLICANT: R Dangarembizi

SCHOOL: Physiology

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *The efficacy and mechanisms of four commercially important phytomedicines on rat models of fever*

Number and Species

130 Sprague Dawley Rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 25 February 2014. This approval remains valid until 24 February 2016.

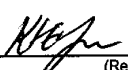
The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: 
(Chairperson, AESC)

Date: 28 March 2014

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: 27/03/2014

cc: Supervisor: Prof K Erlwanger
Director: CAS

Works 2000/1ain0015/AESCcert.wps

APPENDIX 2A: Approved modifications of the study-Rectal thermometry

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND ANIMAL ETHICS SCREENING COMMITTEE MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: DANGAREMBIZI RACHAEL

b. Department: PHYSIOLOGY

c. Experiment to be modified / extended	AESC NO		
Original AESC number	2014	10	C
Other M&Es : N/A			

d. Project Title: The efficacy and mechanisms of four commercially important phytomedicines on rat models of fever.

	No.	Species
e. Number and species of animals originally approved:	130	Rat
f. Number of additional animals previously allocated on M&Es:	N/A	
g. Total number of animals allocated to the experiment to date:	18	
h. Number of animals used to date:	18	

Specific modification / extension requested:

1. To compare core body temperatures recorded using rectal probes with those measured using implanted abdominal data transponders.

Motivation for modification

1. The main objective of our study is to test the efficacy and mechanisms of commercially available phytomedicines as antipyretic agents using rat models of fever. While we will use biotelemetry (a more robust and accurate method for measuring thermal responses) in our study the vast majority of studies published on the antipyretic properties of phytomedicines have shown that most researchers measure febrile responses in rats by recording rectal temperature using rectal probes. We suspect that the process of temperature measurement using rectal probes could result in inaccurate temperature measurements due to the possibility of stress induced hyperthermia. In order for us to see if we can benchmark our responses, measured using biotelemetry, with those from other studies, we will need to test the effects of rectal temperature measurement on both naïve and habituated rats. The measurement of temperatures using rectal probes is a well-established commonly used method but there is no evidence in refereed literature showing the effect of the insertion of the probe on abdominal temperature measurements recorded using biotelemetry

The study will use 40 rats randomly divided into three groups:

- a. Group 1- Naïve non pyretic rats
- b. Group 2- Naïve pyretic rats
- c. Group 3- Habituated non pyretic rats
- d. Group 4- Habituated pyretic rats

Fever will be induced using yeast as described in the approved protocol. We will use surgically implanted data transponders to measure thermal responses to rectal temperature measurement. The protocol for surgical implantation of data transponders will remain unchanged from the approved protocol.

Rectal probe temperature measurements will be taken by gently inserting a vaseline coated probe (external diameter 2 mm) 4 cm into the rectum of the rat (Figuerido et al., 2012) once in every hour for 12 hours (7h00 to 19h00) and this will be done for 3 days (Figuerido et al., 2012; Tomazeti et al., 2005). The depth was chosen to ensure accurate measurements of deep temperatures as it has been shown that temperatures measured close to the anal opening are generally lower and do not correctly represent core body temperatures (Gordon et al., 1990). Minimal restraint will be used in which the rat will be placed on a smooth solid surface, held gently by a hand and its tail lifted before the rectal probe will be inserted (see attached correspondence from Prof Mitchell for detailed guidelines). Approximately 20 s will be required to position the rat for rectal probe insertion and after insertion, the probe will remain in the rectum for 40 s to allow for temperatures to stabilise (Bae et al., 2007). The rat will be allowed to settle and start again if it shows any signs of discomfort and displeasure.

Groups 3 and 4 will be habituated to the temperature measuring procedure for two days before the actual measurement of thermal responses. During habituation, the rats will be subjected to the same temperature measuring procedure as described above twice on each day (Tomazetti et al., 2005).

All other procedures including general care, feeding, monitoring of the health status of the animals, and termination to recover telemeters will also remain unchanged from the approved protocol.

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Date: 11 July 2014

Signature:



RECOMMENDATIONS: Approved. Use of rectal probes to measure temperature in habituated and non-habituated febrile and afebrile rats.

Condition(s): Applicant to be shown how to restrain rats appropriately for rectal thermometry by Prof Duncan Mitchell.

Date: 14 July 2014

Signature:



Chairman, AESC

APPENDIX 2B: Approved modifications of the study-Additional animals for perfusions

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

**UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS**

a. Name: DANGAREMBIZI RACHAEL

b. Department: PHYSIOLOGY

c. Experiment to be modified / extended

AESC NO

Original AESC number	2014	10	C
1 Other M&Es : 2014/10/C			

d. Project Title: The efficacy and mechanisms of four commercially important phytomedicines on rat models of fever.

	No.	Species
e. Number and species of animals originally approved:	130	Rat
f. Number of additional animals previously allocated on M&Es:	N/A	Rat
g. Total number of animals allocated to the experiment to date:	110	Rat
h. Number of animals used to date:	110	Rat

Specific modification/extension requested:

1. Request for allocation of an additional 76 rats to the experiment.
2. Addition of Miss Nyasha Mukonowenzou as an additional co-worker.

Motivation for modification

1.
 - a. We have been characterising yeast induced fevers and the need arose for us to compare temperature measurements from rectal probes with those measured using biotelemetry as described in our M&E (2014/10/C). We allocated 36 rats to the rectal thermometry experiment and these animals were taken from the original number (130 rats) allocated to our study. We are now continuing with the characterisation experiments and need to replace the rats used in the rectal study.
 - b. In our original ethics application (2014/10/C) (Study design included as Fig 1 below), we requested 40 rats for tissue harvesting at 4 time points (rise, peak, fall and end of fever; n=10 per time point) for the yeast-induced fever group. We erroneously omitted the control group with which the febrile animals would be compared. We are therefore requesting for an additional 40 rats (n=10 per time point) to be added as controls at each of the 4 time points.

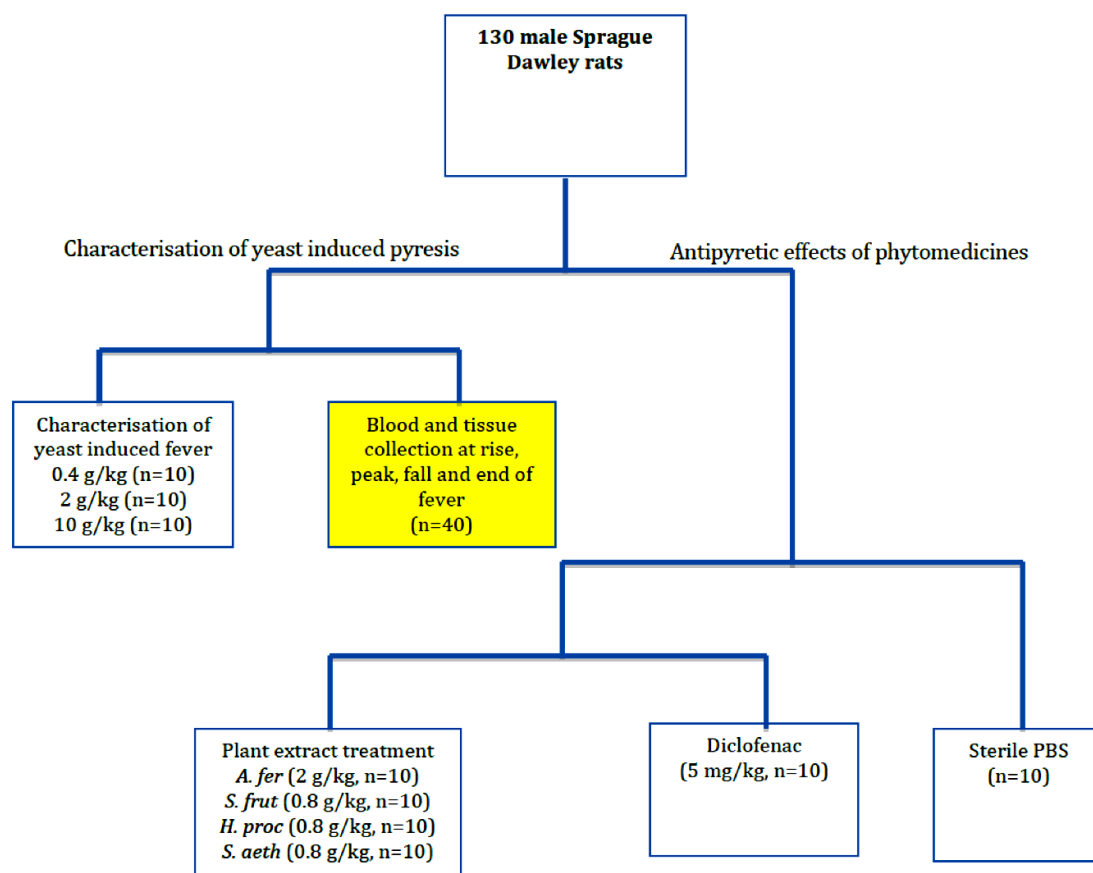


Fig 1: Study design and summary of treatments as requested in 2014/10/C

2. We request to add Miss N. Mukowenzou as a co-worker with because some of the co-workers previously listed have completed their studies and are therefore unavailable to assist. Miss Mukonowenzou will assist with animal handling.

Date: 6/10/2014

Signature:



RECOMMENDATIONS: Approved.

- i. Seventy six additional rats.
- ii. Inclusion of Miss Nyasha Mukonowenzou as a co-worker subject to her attending a CAS first-time user's course.

Date: 7/10/2014

Signature:



Chairman, AESC

APPENDIX 2C: Approved modifications of the study-Use of zymosan as a pyrogen

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND ANIMAL ETHICS SCREENING COMMITTEE MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: DANGAREMBIZI RACHAEL

b. Department: PHYSIOLOGY

c. Experiment to be modified/extended		AESC NO		
Original AESC number	2014	10	C	
Other M&Es : 2014/10/C : A, B, C				

d. Project Title: The efficacy and mechanisms of four commercially important phytomedicines on rat models of fever.

	No.	Species
e. Number and species of animals originally approved:	130	Rat
f. Number of additional animals previously allocated on M&Es:	215	Rat
g. Total number of animals allocated to the experiment to date:	345	Rat
h. Number of animals used to date:	205	Rat

Specific modification / extension requested:

1. Extension of ethics validity
2. To change the experimental pyrogen we use to Zymosan

Motivation for modification

1. Our ethics application (2014/10/C) was valid until the 26th of March 2016 but we are currently in the last phase of our experiments and we would like to request for an extension of the application to the 15th of May 2017.
2. The aim of our study is to determine the efficacy of *Perlaconium sidoides* in attenuating fever. We have to date characterised Brewer's yeast-induced fever and sickness behaviours but the high pyrogenic doses and high volumes of whole yeast cells that are required to induce fever have adverse effects on the rats and usually result in the formation of an abscess which keeps the rats sick for long. We would therefore like to change our experimental pyrogen to zymosan, which is a purified cell wall moiety from *Saccharomyces cerevisiae* which has been shown to induce similar febrile responses at much lower doses and volumes than whole yeast cells (Bastos-Pereira et al., 2014). Zymosan will be injected subcutaneously at three doses, 3 mg, 15 mg and 30 mg. The proposed doses are within the range of doses used in other studies and have not been associated with any adverse effects (Bastos-Pereira et al., 2014, da Silva and Parente, 2001, Ramirez et al., 1999). All procedures for surgery injections and sample collection will remain unchanged and in accordance with our approved protocol.

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Date: 16 May 2016

Signature:



RECOMMENDATIONS: Approved.

- i. Use of Zymosan as the pyrogen.
- ii. Extension of time to 15 May 2017.

Date: 20th May 2016

Signature:



Chairman, AESC

APPENDIX 3A: Published articles-Rectal Thermometry

Physiology & Behavior 179 (2017) 162–167



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Physiology & Behavior

journal homepage: www.elsevier.com/locate/physbeh



Measurement of body temperature in normothermic and febrile rats: Limitations of using rectal thermometry



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ARTICLE INFO

Keywords:

Rectal thermocouple probe
Biotelemetry
Stress-induced hyperthermia
Fever
Brewer's yeast
Radiotelemeters

ABSTRACT

Stress-induced hyperthermia following rectal thermometry is reported in normothermic rats, but appears to be muted or even absent in febrile rats. We therefore investigated whether the use of rectal thermometry affects the accuracy of temperature responses recorded in normothermic and febrile rats. Using intra-abdominally implanted temperature-sensitive radiotelemeters we measured the temperature response to rectal temperature measurement in male Sprague Dawley rats (~200 g) injected subcutaneously with Brewer's yeast (20 ml/kg of a 20% Brewer's yeast solution = 4000 mg/kg) or saline (20 ml/kg of 0.9% saline). Rats had been pre-exposed to, or were naive to rectal temperature measurement before the injection. The first rectal temperature measurement was taken in the plateau phase of the fever (18 h after injection) and at hourly intervals thereafter. In normothermic rats, rectal temperature measurement was associated with an increase in abdominal temperature (0.66 ± 0.27 °C) that had a rapid onset (5–10 min), peaked at 15–20 min and lasted for 35–50 min. The hyperthermic response to rectal temperature measurement was absent in febrile rats. Exposure to rectal temperature measurement on two previous occasions did not reduce the hyperthermia. There was a significant positive linear association between temperatures recorded using the two methods, but the agreement interval identified that rectal temperature measured with a thermocouple probe could either be 0.7 °C greater or 0.5 °C lower than abdominal temperature measured with radiotelemeter. Thus, due to stress-induced hyperthermia, rectal thermometry does not ensure accurate recording of body temperature in short-spaced, intermittent intervals in normothermic and febrile rats.

1. Introduction

Core body temperature has generally been measured in laboratory rodents by inserting a thermocouple probe into the rectum or implanting a radiotelemeter into the abdomen (for review see [1,2]). Even though these two methods potentially yield comparable values [3], one major disadvantage associated with the use of rectal thermometry is that the procedure of handling and inserting the thermocouple probe into the rectum is stressful [4]. The stress associated with rectal temperature measurement leads to a number of physiological changes, which include an increase in body temperature (stress-induced hyperthermia) [5,6] and an increase in neuroendocrine hormones and cytokines [4,7]. While the stress-induced hyperthermia that follows

rectal temperature measurement is commonly reported in normothermic rats, it appears to be muted or even absent in rats with a fever [8].

Despite reports of the procedure causing stress-induced hyperthermia, pharmacological antipyretic testing guidelines continue to advocate the use of rectal thermometry in rats [9,10]. In particular, it is recommended that the test compound or the standard drug be administered 18 h after injection of the pyrogen and rectal temperatures be recorded at regular time intervals thereafter [9,10]. Brewer's yeast is the pyrogen commonly used for inducing pyrexia in pharmacological studies screening antipyretics in rats [9–12]. To determine the efficacy, of the antipyretic pharmacological agents, comparisons are made between the temperatures of treated febrile rats and their normothermic

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counterparts [9,10]. Because the technique used for measuring temperature in these studies appears to cause a prominent increase in body temperature in normothermic, but not febrile rats, it is important to examine whether the use of rectal thermometry could affect the results obtained in these studies.

In an attempt to lessen the stress-induced physiological changes associated with rectal temperature measurement, laboratory animals are often habituated to the procedure before the experiments. Habituation usually involves subjecting the animals to the procedure of rectal temperature measurement for two-to-three days before the start of the experiment [13,14]. Whether this protocol indeed habituates the animals to the procedure of rectal temperature measurement by blunting the stress-induced hyperthermia remains unclear.

Thus, we undertook a study to investigate the effects of rectal temperature measurement on abdominal temperature responses of normothermic and febrile rats, and the effects of commonly used habituation protocols at attenuating the hyperthermic response associated with rectal temperature measurement. We used rats that had either been subjected to rectal temperature measurements before the experiment (pre-exposed), or rats that were naive to rectal temperature measurement. Rats received either saline or a pyrogenic dose of Brewer's yeast. We repeatedly measured rectal temperatures at hourly intervals in febrile rats and normothermic rats while monitoring abdominal temperature changes using radiotelemeters. Our results show that rectal temperature measurement produced a robust, consistent hyperthermic response in normothermic rats, but not in febrile rats during the plateau phase of fever. Pre-exposing normothermic rats to rectal temperature measurements before the experiment did not affect the magnitude or the duration of the hyperthermic response induced by rectal temperature measurements.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats were purchased from the National Health Laboratory Services (Johannesburg, South Africa) and housed in the Central Animal Service Unit of the University of the Witwatersrand, Johannesburg. Rats were housed individually in acrylic cages lined with wood shavings in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) on a 12 h: 12 h light/dark cycle (lights on at 06:00 clock time). Rats were allowed to acclimate to their environment for seven days before surgery. The rats had access to rodent pellets and water ad libitum. On the day of injection, rats had a body mass of between 250 and 300 g; we used non-adult rats so as to follow the published pharmacological antipyretic testing protocol [9,10]. All experiments were performed in accordance with the regulations of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Screening Committee (ethics number 2014/10/C).

2.2. Abdominal temperature

Abdominal temperature was recorded by remote biotelemetry using temperature-sensitive radiotelemeters (TA10TA-F40, Data Sciences, St Paul, MN, USA), which had been implanted intra-abdominally. Rats were anaesthetized with 80 mg/kg ketamine hydrochloride (Anakot-V, Bayer, South Africa) and 20 mg/kg xylazine (Chanazine, Bayer, South Africa). After surgery, the rats received a single intramuscular injection of an analgesic, buprenorphine (Temgesic®, 0.05 mg/kg Schering-Plough, Johannesburg, South Africa) and were given seven days to recover from the surgery before the commencement of the experiments outlined below. Transmitter output frequency (Hz) was monitored at one-minute intervals by a receiver plate (RPC-1, Data Sciences, St Paul, MN, USA) placed beneath the cage of each rat. The frequency received by each plate was fed into a peripheral processor connected to a personal computer and converted to degrees Celsius. The radiotelemeters

were calibrated individually by the manufacturer (Data Sciences, St Paul, MN, USA), in a water bath at 35°C and 39°C , to an accuracy of 0.1°C .

2.3. Rectal temperature

Rectal temperature measurements were obtained by gently inserting a lubricated thermocouple probe (Type T copper/constantan thermocouple probe, external diameter 2 mm) 40 mm into the rectum of each rat. An insertion depth of 40 mm has been used successfully for determining core body temperature in rats [5,15,16]. The polyvinyl chloride-insulated thermocouple probe was calibrated against a high-accuracy thermometer (Quat 100, Heraeus, Hanau, Germany) in an insulated water bath across a range of temperatures ($35, 36, 37, 38, 39$ and 40°C) to an accuracy of 0.1°C . To measure rectal temperature the rat was placed with all four paws on a smooth, solid surface and gently held in place by a hand positioned over the back, while the tail was lifted and the thermocouple probe was inserted. It took time (30–120 s) to settle the rats and position them for the insertion of the thermocouple probe into the rectum. Once inserted the thermocouple probe remained in the rectum for 40 s to allow temperatures to stabilise [5]. All rectal temperature measurements were performed by one person (Rachael Dangarembizi).

2.4. Pyrogen

Brewer's yeast (Sigma Aldrich, St Louis, USA: Lot# SLBC 5519 V) was reconstituted in sterile, non-pyrogenic 0.9% saline (Sabax, Johannesburg, South Africa) to a final concentration of 20% (w/v) and the yeast solution was injected subcutaneously (s.c.) in the dorsum of the neck at a volume of 20 ml/kg (4000 mg/kg) [17,18].

2.5. Experimental procedures

To determine if pre-exposure to the procedure of rectal temperature measurement reduced the magnitude of stress-induced hyperthermia, we measured the rectal temperature of rats once a day (1–2 h before the onset of the dark phase) for two days before they received injections. We called these rats "pre-exposed", while the other rats that received their first rectal temperature measurement only on the day of injections were called "naive". Pre-exposed and naive rats received an s.c. injection of either Brewer's yeast or saline. As additional controls, another group of twelve rats received an s.c. injection of Brewer's yeast, but without subsequent rectal temperature measurements. In an attempt to reduce the number of animals used for the study we chose not to include an additional group of rats that received saline followed by no rectal temperature measurements (referred to in Figs. 1 and 3B as "no rectal probe"), but rather to use abdominal temperature recordings from pre-intervention days when the rats were left undisturbed in their home cages. The final experimental groups are summarized in Table 1.

Injections were administered at 16:00, 2 h before the onset of the dark phase. Pharmacological antipyretic testing guidelines in rats recommend that the test compound or the standard drug be administered 18 h after injection of the pyrogen and rectal temperatures be recorded at regular time intervals of 30–60 min for 5 h thereafter. We therefore started our rectal temperature measurements 18 h after injection of Brewer's yeast and rectal temperature measurements were taken once every hour, over a five-hour period (10:00–15:00) [9,10]. Following each rectal temperature measurement rats were returned to their home cages.

2.6. Data analysis

All data were analyzed using GraphPad Prism software (version 7.0, GraphPad Software Inc., USA). Statistical significance was accepted for $P < 0.05$. To analyze the fever induced by s.c. administration of

Table 1
Summary of experimental interventions and sample size.

Intervention	Sample size
Rectal probe pre-exposed + Brewer's yeast	9 (2 rats excluded in final analysis)
Rectal probe naive + Brewer's yeast	9 (1 rat excluded in final analysis)
Rectal probe pre-exposed + Saline ^a	8
Rectal probe naive + Saline ^a	9 (1 rat excluded in final analysis)
No rectal probe + Brewer's yeast (6 of the 12 rats where used for calculating the "baseline" average abdominal temperatures of febrile rats as explain in the data analysis section).	12

^a In an attempt to reduce the number of animals used for the study we chose not to include an additional group of rats that received saline followed by no rectal temperature measurements (referred to in Figs. 1 and 3B as "no rectal probe"), but rather to use abdominal temperature recordings from pre-intervention days when these rats were left undisturbed.

Brewer's yeast, the original one-minute abdominal temperature recordings were averaged over 2 h and the two-hourly means compared by repeated measures two-way analysis of variance (ANOVA) with intervention and time as main effects. Tukey's post hoc test was used to detect differences between groups when the ANOVA detected significant main effects or interactions.

We performed Pearson's correlation analyses to calculate the correlation coefficient (r) between rectal temperatures recorded using a thermocouple probe and abdominal temperatures recorded using a radiotelemeter. To determine the level of agreement between the two methods of core body temperature measurement we performed a Bland-Altman analysis [19].

As mentioned earlier, a group of rats ($n = 12$) received an s.c. injection of Brewer's yeast, but without subsequent rectal temperature measurements. We used six of these rats to calculate the "baseline" average abdominal temperatures of febrile rats 18–23 h after injection of Brewer's yeast, i.e. during the five-hour experimental period (10:00–15:00). To determine the effect of rectal temperature measurements on the abdominal temperature of febrile rats, we calculated the difference between the abdominal temperatures of rats having or not having rectal temperature measurements and the baseline average abdominal temperatures calculated as outlined above. Rats were excluded from data analysis when temperature responses differed by more than twice the standard deviation of the group mean ($n = 2$ Rectal probe pre-exposed + Brewer's yeast; $n = 1$ Rectal probe naive + Brewer's yeast).

To determine the effect of rectal temperature measurements on the abdominal temperature of normothermic rats over the five-hour experimental period (10:00–15:00), we calculated the difference between the abdominal temperatures of a rat having rectal temperature measurements following injection of saline or not having rectal temperature measurements from the average abdominal temperature of the rat when left undisturbed at exactly the same time of the day on three previous days. For statistical purposes, the one-minute changes in abdominal temperature values, calculated as outlined above, were averaged over 5 min for the five-hour experimental period (10:00–15:00). The five-minute means were compared by repeated measures two-way ANOVA with intervention and time as main effects. Tukey's post hoc test was used to detect differences between groups when the ANOVA detected significant main effects or interactions. Rats were excluded from data analysis when temperature responses differed by more than twice the standard deviation of the group mean ($n = 1$ Rectal probe naive + Saline).

The maximum change in abdominal temperature, after each rectal temperature measurement in pre-exposed and naive rats that received saline, was regressed linearly against the number of rectal temperature

measurements for each rat to determine if normothermic rats habituate to five repeated measurements of rectal temperatures spaced an hour apart.

3. Results

Fig. 1 shows that rats injected s.c. with Brewer's yeast initially had a significant decrease in abdominal temperature ($36.81 \pm 0.35^\circ\text{C}$) 2 h after injection compared to rats injected with saline ($37.56 \pm 0.23^\circ\text{C}$). Following the brief period of hypothermia, the abdominal temperature of rats injected with Brewer's yeast started to increase and peaked at $39.02 \pm 0.3^\circ\text{C}$ eight hours after injection and remained significantly elevated for 72 h compared to rats injected with saline (main effects of time ($F_{(36, 1692)} = 212.8$, $P < 0.0001$), treatment ($F_{(5, 47)} = 149$, $P < 0.0001$) and their interaction ($F_{(180, 1692)} = 29.71$, $P < 0.0001$)).

The difference in the core body temperature between rats injected with Brewer's yeast and rats injected with saline represents the amplitude of the fever. Greater fever amplitudes were noted at 18 h after injection (the time at which fever is usually assessed in pharmacological antipyretic screening studies) with temperature values obtained from radiotelemeters implanted in the abdomen (mean difference 1.79°C and 95% confidence intervals 1.56 and 2.03°C), than with temperature values obtained from thermocouple probes inserted into the rectum (mean difference 1.56°C and 95% confidence intervals 1.31 and 1.8°C).

The rectal temperature and abdominal temperature values measured at 18 h after injection were significantly correlated in rats that received saline ($r = 0.79$, $P = 0.0003$) and in rats that received Brewer's yeast ($r = 0.64$, $P = 0.01$). Fig. 2 shows the mean difference (bias) and limits of agreements of the rectal temperatures and abdominal temperatures measured at 18 h after injection. The temperature values measured with the two methods differed in rats injected with

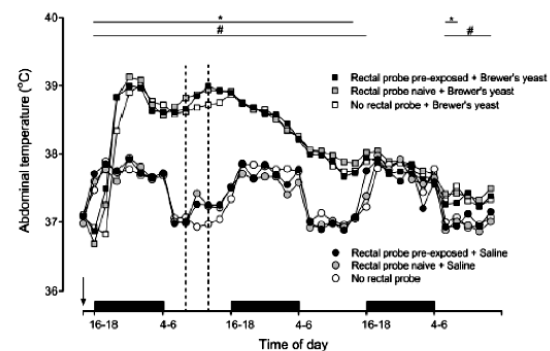


Fig. 1. Abdominal temperature (mean and SD) of rats injected s.c. with Brewer's yeast (20 ml/kg of a 20% Brewer's yeast solution = 4000 mg/kg) or saline (20 ml/kg of 0.9% saline). Pre-exposed rats had rectal temperature measurements taken once a day on the two days before receiving an injection of Brewer's yeast ($n = 7$) or saline ($n = 8$). Naive rats received the first rectal temperature measurement 18 h after receiving injections of either Brewer's yeast ($n = 8$) or saline ($n = 8$). The arrow indicates the time of injection and dashed lines indicate the period during which rectal temperature measurements were taken (10:00–15:00 clock time on day 1 post-injection). Brewer's yeast and no rectal probe represent rats that received an injection but no rectal temperature measurements ($n = 6$). No rectal probe represents the abdominal temperature when the rats that received saline were left undisturbed in their home cages; i.e. before exposure to rectal temperature measurements started. As there were no significant differences in the temperature profiles obtained for the naive and pre-exposed rats during the days when they were not disturbed, the data for both groups were combined to create one temperature profile for the no rectal probe data ($n = 16$). The original one-minute abdominal temperature recordings were averaged over 2 h and the two-hourly means have been plotted. *Rectal probe pre-exposed + Brewer's yeast significantly different to Rectal probe pre-exposed + Saline (Tukey's, $P < 0.05$). #Rectal probe naive + Brewer's yeast significantly different to Rectal probe naive + Saline (Tukey's, $P < 0.01$).

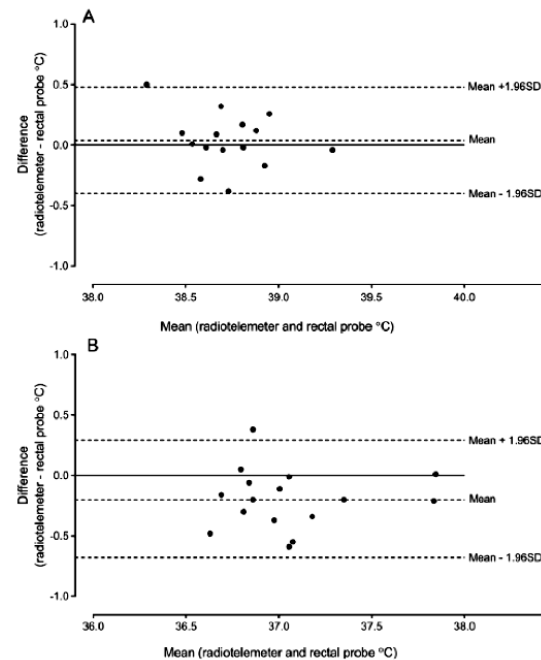


Fig. 2. The mean difference (bias) and limits of agreements between the rectal temperatures and abdominal temperatures measured at 18 h in (A) rats with a fever induced by Brewer's yeast ($n = 15$) or (B) rats injected with saline ($n = 16$). Rats were housed at an ambient temperature of $22 \pm 2^\circ\text{C}$. Black dots represent individual animals. Upper limit of agreement is calculated as the mean difference between the two methods plus $1.96 \times \text{SD}$ of the difference. Lower limit of agreement is calculated as the mean difference between the two methods minus $1.96 \times \text{SD}$ of the difference.

Brewer's yeast (mean difference 0.04 ± 0.23 ; 95% limits of agreement -0.4 to 0.48) and in rats injected with saline (mean difference -0.2 ± 0.25 ; 95% limits of agreement -0.68 to 0.29). Thus the agreement interval between the two methods identified that the temperature values recorded in rats using a thermocouple probe inserted into the rectum could either be 0.7°C greater or 0.5°C lower than those obtained with a radiotransmitter implanted into the abdomen.

Fig. 3A shows that the average change in abdominal temperature, over the 5-hour period (10:00–15:00), of rats pre-exposed or naive to rectal temperature measurements and injected s.c. with Brewer's yeast was not significantly different from the change in abdominal temperature of rats not exposed to rectal temperature measurements and injected with Brewer's yeast (main effects of time ($F_{(59, 1062)} = 4.04$, $P < 0.0001$), treatment ($F_{(2, 18)} = 2.63$, $P = 0.10$) and their interaction ($F_{(118, 1062)} = 1$, $P = 0.49$)).

Rectal temperature measurement induced a significant increase in the abdominal temperature of rats injected s.c. with saline compared to when they did not receive rectal temperature measurements, i.e. when they were left undisturbed in their home cages (Fig. 3B) (main effects of time ($F_{(59, 1711)} = 20.49$, $P < 0.0001$), treatment ($F_{(2, 29)} = 20.42$, $P < 0.0001$) and their interaction ($F_{(118, 1711)} = 7.02$, $P < 0.0001$)). After each rectal temperature measurement, the increase in abdominal temperature, of rats injected with saline that were pre-exposed or naive to rectal temperature measurements, occurred after a latent period of 5–10 min, peaked at 15–20 min with a mean maximum change of $0.66 \pm 0.27^\circ\text{C}$, and resolved after 35–50 min. The regression slopes plotted for the maximum change in abdominal temperature, recorded after each rectal temperature measurement for each rat that received saline, against number of rectal temperature measurements

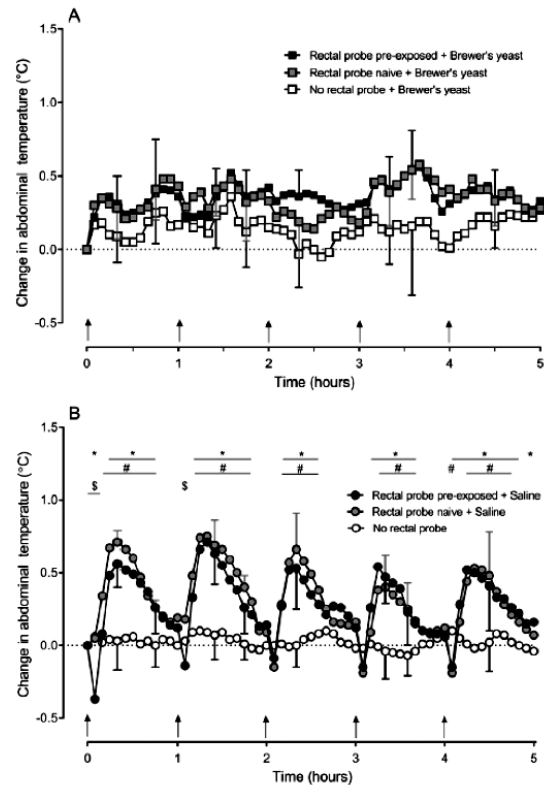


Fig. 3. Change in abdominal temperature (mean and SD), shown in five-minute intervals over 5 h following rectal temperature measurement in (A) febrile rats injected s.c. with Brewer's yeast (20 ml/kg of a 20% Brewer's yeast solution = 4000 mg/kg) ($n = 7$ pre-exposed; $n = 8$ naive) and (B) normothermic rats injected s.c. with saline (20 ml/kg of 0.9% saline) ($n = 8$ pre-exposed; $n = 8$ naive). Pre-exposed rats had rectal temperatures taken once a day on the two days before receiving an injection. Naive rats received their first rectal temperature measurement only at T_0 which corresponded to 18 h after the injections. $T_0 = 10:00$ clock time and $T_5 = 15:00$ clock time. The black arrows indicate the time of handling and rectal temperature measurements. Brewer's yeast and no rectal probe represent rats that received an injection but no rectal temperature measurements ($n = 6$). No rectal probe (B) represents the change in abdominal temperature when the rats that received saline were left undisturbed in their home cages; i.e. before exposure to rectal temperature measurements started. As there were no significant differences in the temperature profiles obtained for the naive and pre-exposed rats during the days when they were not disturbed, the data for both groups were combined to create one temperature profile for the no rectal probe data ($n = 16$). *Rectal probe pre-exposed + Saline significantly different to no rectal probe (Tukey's, $P < 0.05$). #Rectal probe naive + Saline significantly different to no rectal probe (Tukey's, $P < 0.05$). \$Rectal probe pre-exposed + Saline significantly different to Rectal probe naive + saline (Tukey's, $P < 0.05$). No significant differences were observed between the febrile groups.

significantly deviated from zero in only three of the sixteen rats. Thus, for most rats, the maximum change in abdominal temperature induced by rectal temperature measurement did not decrease with repeated rectal temperature measurements taken at hourly intervals.

4. Discussion

We have shown that the procedure of measuring rectal temperature induces an increase in abdominal temperature ($0.66 \pm 0.27^\circ\text{C}$) in normothermic rats, but not in febrile rats during the plateau phase of fever (Fig. 3). In normothermic rats, the stress-induced hyperthermia observed in our study had a rapid onset (within 5–10 min), peaked at

15–20 min and lasted for 35–50 min after each rectal temperature measurement (Fig. 3B). The exposure of rats to the procedure of rectal temperature measurement on two occasions before the start of the experiment, and five repeated rectal temperature measurements undertaken on the day of the experiment, did not significantly reduce the magnitude or duration of the stress-induced hyperthermia (Fig. 3B). The difference in temperature between rats injected with saline vs rats injected with Brewer's yeast, i.e. the amplitude of the fever, was smaller when rectal temperature was measured, than when abdominal temperature was measured. There was a significant positive linear association between rectal temperature and abdominal temperature in normothermic and febrile rats. Overall, the agreement interval between the two methods identified that rectal temperature measured with a thermocouple probe could either be 0.7 °C greater or 0.5 °C lower than abdominal temperature measured with radiotelemetry (Fig. 2).

Our findings have important implications for the use of rectal thermometry to measure core body temperature in pharmacological antipyretic screening studies. In these studies, the rats are usually injected with either Brewer's yeast or saline followed by the antipyretic agent being tested [17,18,20]. The efficacy of the antipyretic agent is determined by comparing the rectal temperatures of the treated febrile rats and the normothermic controls. Similar core body temperature measurements in the treated febrile rats and normothermic rats would indicate that the antipyretic agent being tested is indeed effective. Our results show that rectal temperature measurements underestimated the amplitude of fever and normothermic rats injected with saline have a significant increase in abdominal temperature for 5–50 min after each rectal temperature measurement (Fig. 3B). Thus, the use of rectal thermometry may potentially lead to investigators erroneously reporting an antipyretic effect of the agent under investigation, if the rectal temperature measurements are taken at intervals of < 60 min, which has been reported in some studies (for example see [12,20–22]). The majority of the pharmacological antipyretic screening studies however record rectal temperatures in hourly intervals. As shown in our study, the hyperthermic response resolves between 40 and 50 min after rectal temperature measurement, hence values measured at hourly intervals are less likely to reflect the stress-induced hyperthermia induced by the rectal temperature measurement. Others have identified that the increase in corticosterone following rectal temperature measurement also resolves within 60 min after rectal temperature measurement [4]. Rectal temperature measurement is also associated with a concomitant rapid increase in serum concentrations of the pro-inflammatory cytokines IL-1 β and IL-6 [7]. Therefore, it is important to note that, even if the stress-induced hyperthermia resolves within 60 min, the basic biochemical make-up and physiological responses of a stressed rat will be different from those of an undisturbed rat.

In our study, subjecting the rats to rectal temperature measurements on two previous occasions before the start of the experiment, as is commonly done in other studies using rectal thermometry [13,14], did not change the magnitude or duration of the hyperthermia induced by the rectal temperature measurements. Although we did not strictly investigate what length of habituation would be required to decrease the hyperthermic response noted with rectal temperature measurement, two days before the experiment may be too short a period to pre-expose the rats and habituate them to the procedure. In support of our observation, others have also reported no reduction in the hyperthermic response of rats pre-exposed to rectal temperature measurement 15 times over a period of five days [5].

Overall, the agreement interval between the two methods identified that rectal temperature measured with a thermocouple probe could either be as much as 0.7 °C greater or 0.5 °C lower than abdominal temperature measured with a radiotelemetry (Fig. 2). Other researchers have reported mean differences in excess of 1 °C in normothermic rats [3]. The accuracy of rectal temperatures recorded using a thermocouple probe is heavily dependent on the probe insertion depth; temperatures measured closer to the anal opening (further away for the liver) are

lower and a less accurate representation of core body temperature. Thus, the accuracy of the rectal temperature measurements recorded using a thermocouple probe is contingent on the technique and consistency of the researcher. These factors are however difficult to standardize because not all animals cooperate or respond in the same way. In this regard, although surgically implanted radiotelemeters are more expensive than thermocouple probes, they appear to be a more reliable method of core body temperature measurement in rodents, particularly in studies measuring temperature responses over a prolonged period of time [23].

Our study has shown that while the hyperthermic response to rectal temperature measurement is prominent, long-lasting and reproducible in normothermic rats (Fig. 3B) it appears absent in febrile rats during the plateau phase of fever induced by s.c. administration of Brewer's yeast (Fig. 3A). Results obtained from studies using other stressful events, such as cage-switching and exposure to an open field, have reported conflicting findings regarding the possible involvement of prostaglandins, key mediators of fever, in stress-induced hyperthermia [8,24–28]. If prostaglandins are indeed involved in mediating stress-induced hyperthermia then the absence of hyperthermia following rectal temperature measurement in febrile rats might be related to saturation of prostaglandin receptors. Alternatively it has previously been mooted that the absence of stress-induced hyperthermia in febrile rats is related to a ceiling effect i.e. a maximal reachable temperature above which no further rise is possible [8]. It is unlikely that a ceiling effect for body temperature contributed to the absence of the hyperthermia observed in febrile rats in our study, as the rats had attained greater temperatures during the peak of the fever eight-to-ten hours before the start of the rectal temperature measurements (Fig. 1).

The proposed sources of heat production associated with stress-induced hyperthermia include: (1) increased non-shivering thermogenesis in brown adipose tissue (BAT); (2) cutaneous vasoconstriction to reduce heat loss (mediated by sympathetic outflow especially to the tail) and (3) activation of the hypothalamic-pituitary-adrenal (HPA) axis to increase production of thermogenic hormones [29–32]. In our study, rats were housed in a subthermoneutral environment (22 ± 2 °C), and at these temperatures, the aforementioned heat generating mechanisms are required to maintain normal core body temperature. The same heat generating mechanisms are also employed in maintaining the febrile state in rats. The “law of initial values” postulated by Wilder in 1931 [33], states that the direction and magnitude of a physiological response depends, to a great extent, on the initial (pre-stimulus) values. Thus the existence of elevated initial (pre-stimulus) values may constrain further increases in a physiological response and therefore lessen the magnitude of the response observed when a stimulus is applied. Applying this law to our findings, the absence of the stress-induced hyperthermia in the febrile rats in our study may be related to a pre-existent background activation of heat generating mechanisms for cold defense and maintenance of fever. In support of this hypothesis, is the finding by others that febrile rats showed a mild, but noticeable hyperthermia when rectal temperature was measured during the defervescence phase of fever, when autonomic responses e.g. vasoconstriction and non-shivering thermogenesis are reduced [8].

In conclusion, we have shown that the procedure of rectal temperature measurement induces a clear hyperthermic response in normothermic rats, but not in febrile rats during the plateau phase of fever. Our findings are important particularly for antipyretic screening studies where conclusions are drawn based on comparisons of temperature responses of febrile rats and their normothermic controls. In these studies the use of rectal thermometry may affect the reliability of results especially if short-spaced, intermittent rectal temperature measurements are used.

Conflict of interest

All authors declare no conflict of interest.

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APPENDIX 3: Published articles- Brewer's yeast is. A potent inducer of fever, sickness behaviour and inflammation within the brain

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Full-length Article

Brewer's yeast is a potent inducer of fever, sickness behavior and inflammation within the brain



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Systemic inflammation

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Anorexia

Lethargy

Circumventricular organs

ABSTRACT

Brewer's yeast, derived from the yeast species *Saccharomyces cerevisiae* (*S. cerevisiae*), is commonly used for inducing pyrexia in pharmacological studies screening antipyretics in rats. Despite its widespread use, the peripheral and central inflammatory response associated with Brewer's yeast-induced fever and sickness behavior in rats has not been investigated. Thus, we injected male Sprague-Dawley rats (150–200 g) subcutaneously with a high (4 g/kg, *n* = 9), medium (2 g/kg, *n* = 5) or low (0.4 g/kg, *n* = 6) dose of Brewer's yeast solution or saline (0.9%, *n* = 6) and measured core body temperature, cage activity, food intake and body mass for six days after injection. Blood and brain samples were collected at 2, 8, 18 and 72 h after injection; *n* = 5–7 per time point. Brewer's yeast administration dose-dependently induced fever, lethargy, anorexia and body mass stunting that was accompanied by increased blood plasma levels of interleukin (IL)-6 and tumor necrosis factor (TNF)- α and activation of inflammatory transcription factors (nuclear factor (NF) for interleukin-6, signal transducer and activator of transcription (STAT)-3, and NF- κ B) in the hypothalamus and circumventricular organs. The increased activation of transcription factors following Brewer's yeast administration was accompanied by increased hypothalamic mRNA expression of TNF- α , IL-1 β and IL-6 and rate-limiting enzymes for prostaglandin synthesis. Our results show that subcutaneous administration of *S. cerevisiae* induces prolonged fever, anorexia and lethargy that is accompanied by a pronounced increase in the synthesis of pro-inflammatory cytokines, key prostaglandin synthesizing enzymes and transcription factors, in the periphery and brain.

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1. Introduction

For almost a century, brewer's/baker's yeast derived from the yeast species *Saccharomyces cerevisiae* (*S. cerevisiae*) has remained

a commonly used fungal pyrogen for inducing pyrexia in pharmacological, especially ethnopharmacological, studies screening antipyretics in rats (Nicolle, 1925; Tomazetti et al., 2005; Loux et al., 1972; Aouey et al., 2016; Abotsi et al., 2016; Liu et al., 2016). The pharmacological guidelines for testing antipyretics in rats recommend that the test compound or the standard drug be administered 18 h after injection of yeast and rectal temperatures be recorded at intervals of 30–60 min for five hours thereafter (Thangaraj, 2016; Vogel, 2002). Results obtained from studies describing the time-dependent changes in core body temperature induced in rats injected with brewer's yeast (1 g/kg–4 g/kg), have identified that the recommended drug administration time-point of 18 h is likely to coincide with the stable or plateau phase of the fever, i.e. an appropriate time to test antipyretic activity of drugs (Brugueroles and Roucoules, 1994; Dangarembizi et al., 2017; Kent et al., 1988). Although the fever pattern accompanying brewer's yeast administration in rats has been described, the

Abbreviations: COX, cyclooxygenase; DAPI, 4,6-diamidino-2-phenylindole; EDTA, ethylenediamine tetraacetic acid; I κ B α , nuclear factor kappa light polypeptide gene enhancer in B cells inhibitor; LPS, lipopolysaccharide; MnPO, median preoptic nucleus; mPGES, microsomal prostaglandin G synthase; MPO, mouse leukemia virus; NF- κ B, nuclear factor kappa light polypeptide gene enhancer in B cells; NF, IL-6 – nuclear factor for interleukin-6; OVLT, vascular organ of the lamina terminalis; IL, interleukin; PG, prostaglandin; RTPCR, Real Time Polymerase Chain Reaction; STAT-3, signal transducer and activator of transcription-3; SOCS-3, suppressor of cytokine signaling; TNF, tumor necrosis factor; NDS, normal donkey serum; vWf, von Willebrand factor.

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inflammatory profile, in the periphery and brain, accompanying the time-dependent changes in core body temperature has not been fully described. Thus, it is unclear which inflammatory mediators are present at and beyond the recommended antipyretic administration time point of 18 h.

Mechanisms of action of currently used antipyretics include: (1) inhibiting the enzyme cyclooxygenase (COX) and reducing the concentration of prostaglandin (PG) E_2 within the hypothalamus, (2) reducing pro-inflammatory mediators, and (3) enhancing anti-inflammatory signals at the site of infection and within the brain (Aronoff and Neilson, 2001). The activation of transcription factors controls the expression of immune response genes that ultimately leads to the synthesis and release of the inflammatory mediators regulating fever (for review see Rummel (2016)). The most important inflammatory transcription factors implicated in regulating the inflammatory response associated with fever include: nuclear factor (NF)- κ B, NF-IL6, signal transducer and activator of transcription (STAT) 3 and suppressor of cytokine signaling (SOCS)-3, the endogenous negative inhibitor of STAT-3 signaling (for review see Rummel (2016)). Thus, concurrently describing the fever-pattern and the inflammatory mediators and inflammatory transcription factors induced following yeast administration would provide valuable insight for studies using brewer's yeast to screen antipyretics and determine their mechanism of action.

Determining the inflammatory response to brewer's yeast would not only be beneficial for studies using brewer's yeast to screen antipyretics, but it would also improve our understanding of the interaction between fungal pathogens and the immune system. The increased prevalence of human infections associated with *S. cerevisiae* and the large number of *S. cerevisiae* viable cells in commercial and food-related products has increased the interest in studying yeast-immune system interaction (De Llanos et al., 2006; Riquelme et al., 2003; Souza et al., 2012). Using zymosan, a cell wall extract of *S. cerevisiae*, studies have shed some light on possible yeast-immune interactions and shown that it can directly stimulate immune cells, such as macrophages (Sorenson et al., 1998) and the key brain area involved in regulating fever, namely the median preoptic nucleus (MnPO) of the hypothalamus (Bastos-Pereira et al., 2014). Moreover, the finding that zymosan can induce the release of pro-inflammatory cytokines in the vascular organ of the laminae terminalis (OVLT), one of the three sensory circumventricular organs lacking a tight-blood brain barrier, also highlights a potential yeast-immune-to-brain communication pathway (Bastos-Pereira et al., 2014).

Thus, we have chosen to determine the inflammatory response, in the periphery and brain, induced by subcutaneous administration of brewer's yeast. Pharmacological antipyretic testing guidelines recommend administering brewer's yeast subcutaneously (Thangaraj, 2016; Vogel, 2002). We measured key pro- and anti-inflammatory mediators identified to be involved in regulating fever and sickness behavior responses, namely: transcription factors (NF-IL6, NF- κ B, STAT-3 and SOCS-3), pro-inflammatory cytokines (interleukin (IL)-6, IL-1 β , tumour necrosis factor (TNF)- α) and enzymes involved in $PG E_2$ synthesis (COX-2 and microsomal prostaglandin E synthase (mPGES)). In terms of the inflammatory response in the brain, we focused on brain endothelial cells and key brain regions implicated in mediating fever, namely the hypothalamus and OVLT. Our rationale for focusing on endothelial cells is that one of primary mechanisms by which pro-inflammatory cytokines elicits fever is by inducing $PG E_2$ synthesis in brain endothelial cells (Eskilsson et al., 2014). In addition to measuring the inflammatory response to brewer's yeast administration we also measured changes in body temperature, cage activity, food intake and body mass, so as to concurrently describe the time-dependent inflammatory and sickness responses that accompany brewer's yeast administration. Our results show that brewer's

yeast administered subcutaneously, at doses of 4 g/kg and 2 g/kg, is a potent inducer of fever, lethargy, anorexia and growth stunting. Overall the most prominent increase in the synthesis of pro-inflammatory cytokines, key prostaglandin synthesizing enzymes and transcription factors, in the periphery and brain, coincided with the fever peak, 8 h after yeast administration. Inflammatory responses were muted in the periphery and the brain, at and beyond 18 h, after brewer's yeast administration.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (initial body mass 100–150 g) were purchased from the National Health Laboratory Service (Johannesburg, South Africa) and housed in the Central Animal Service Unit of the University of the Witwatersrand, Johannesburg. The rats were housed individually in acrylic cages lined with wood shavings in a temperature-controlled room (ambient temperature of $22 \pm 2^\circ\text{C}$) on a 12 h:12 h light/dark cycle (lights on at 06:00 clock time). Food (pelleted rat chow, Epol, Johannesburg, South Africa) and water were provided *ad libitum*. The rats were allowed to acclimate to their environment for seven days before surgery. On the day of injection of the pyrogen, rats had a body mass of between 250 and 300 g. All experiments were performed in accordance with the principles of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Screening Committee (ethics clearance number 2014/10/C).

2.2. Pyrogen administration

Rats received a single subcutaneous (s.c.) injection of brewer's yeast (Sigma Aldrich, St Louis, USA: lot number SLBC 5519V) reconstituted in a vehicle of 0.9% sterile, non-pyrogenic saline (Sabax, Johannesburg, South Africa) to a final concentration of 20% (w/v). The yeast solution was injected in the dorsum of the neck at a volume of 20 ml/kg (4 g/kg), 10 ml/kg (2 g/kg) or 2 ml/kg (0.4 g/kg). The 4 g/kg and 2 g/kg doses of brewer's yeast are commonly used in pharmacological studies for inducing fever in young rats (~200–250 g) (Keri et al., 2010; Zakaria et al., 2006; Ghule et al., 2007).

2.3. Body temperature and cage activity

As previously described by Hopwood et al. (2009), we measured core body temperature and cage activity of rats using sterile, wax-coated temperature-sensitive radiotelemeters (TA10TA-F40, Data Sciences, St Paul, MN, USA), that had been implanted intraperitoneally, with rats under general anesthesia induced by an intramuscular injection of ketamine hydrochloride (80 mg/kg, Anakot-V, Bayer, South Africa) and xylazine (20 mg/kg, Chanazine, Bayer, South Africa). After surgery the rats received a single intramuscular injection of an analgesic (Temgesic®; Schering-Plough, Johannesburg, South Africa), and were then given a week to recover from the surgery. The radiotelemeters were calibrated in a water bath at 35°C and 39°C by the manufacturer (Data Sciences, St Paul, MN, USA) to an accuracy of 0.1°C .

2.4. Body mass and food intake

We measured body mass and food intake daily, two hours before the beginning of the dark phase of the circadian cycle, using an electronic scale (Diamond, US) accurate to one gram. To calculate daily food intake we gave each rat 200 g of standard rat chow and subtracted the weight of the food remaining after 24 h from

the initial weight of food (200 g). We corrected for food remaining on the cage floor.

2.5. IL-6 and TNF- α bioassays

As described previously by Ott et al. (2010) we performed a bioassay based on the dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line (Aarden et al., 1987) and on the cytotoxic effect of TNF- α on a mouse fibrosarcoma cell line WEHI 164 subclone 13 (Espvik and Nissen-Meyer, 1986) to determine the level of IL-6 and TNF- α in plasma. The detection limit of the assay, after considering the dilution of samples for the assays, proved to be 3 international units (IU) for IL-6/ml and 6 pg/ml for TNF- α .

2.6. Tissue preparation

For immunohistochemical analysis, we cut coronal brain sections (20 μ m) at -20°C on a cryostat (2800 Frigocut-E, Cambridge Instruments, Germany) and thaw mounted the sections onto poly-L-lysine-coated glass slides. The brain sections we used in this study were prepared from areas encompassing the vascular organ of the lamina terminalis (OVL) (0.40–0.60 mm bregma) according to the stereotaxic atlas of the rat brain (Paxinos and Watson, 1998). In total, we collected twelve coronal brain sections per rat. For RT-PCR analysis we used the same brains as used for immunohistochemical analysis and thaw-mounted 35 consecutive frozen 30 μ m sections onto glass slides from which we dissected out the hypothalamus for RNA extraction. We stored the samples at -55°C until processing.

2.7. Immunohistochemistry

2.7.1. General procedure

We air dried the frozen brain sections for 10 min at room temperature and then fixed them in 2% paraformaldehyde (PFA; Merck, Darmstadt, Germany) diluted in phosphate buffered saline (PBS). Thereafter, we washed the sections in PBS for 5-min, three times and then treated the sections for one hour at room temperature with a blocking solution consisting of 10% normal donkey serum (NDS; Biozol, Germany) and Triton X-100 (0.1% for NF-IL6 and NF- κ B, and 0.3% for COX-2 and STAT-3). Table 1 shows the details of the primary antibody dilutions. We incubated the sections with the primary antibodies for 20–22 h at 4°C . We have previously tested the specificity of the respective antibodies (Damm et al., 2011; Fuchs et al., 2013; Rummel et al., 2006; Rummel et al., 2011). After three 5-min washes in PBS, we incubated the sections with secondary antibodies conjugated with fluorescent dyes for two hours at room temperature (Table 1 shows the details of the

secondary antibody dilutions). We counterstained the sections with nuclear 4',6'-diamidino-2-phenylindole (DAPI) stain (1:8000 dilution in PBS; Merck, Darmstadt, Germany) for 10 min to clearly visualize the tissue surrounding the immunoreactive signal and to demonstrate the nuclear localization of the signal. Finally, we cover-slipped (glass covers) all the sections with a glycerol/PBS solution (Citifluor, Ltd, London, UK) and stored the sections at 4°C until microscopic analysis was performed.

2.7.2. Colocalization

Double immunohistochemistry was performed to determine the contribution of endothelial cells to the NF-IL6, NF- κ B, STAT-3 and COX-2 immunoreactive cell population. Before the first incubation, the antibodies for NF-IL6, NF- κ B, STAT-3 or COX-2 were mixed with the antibodies for von Willebrand factor, a marker for endothelial cells (dilutions are given in Table 1). NF-IL6, NF- κ B and STAT-3 were visualized with Cy3-conjugated anti-rabbit IgG and COX-2 was visualized with Cy3-conjugated anti-goat IgG (dilutions given in Table 1). Endothelial cell markers were visualized with Alexa-488-conjugated anti-rabbit or anti-sheep IgG (dilutions given in Table 1). In control experiments, the sections were incubated with the primary antibody mixture in which one of the primary antibodies was substituted with nonimmunized animal IgG (goat IgG for COX-2, rabbit IgG for NF-IL6, NF- κ B and STAT-3). Inappropriate cross-reaction between antibodies was confirmed to be negligible.

2.8. Microscopic analysis

We acquired the images using a light/fluorescent microscope (Olympus BX50, Olympus Optical, Hamburg, Germany) with a black and white Spot Insight camera (Diagnostic Instruments, Vision Systems, Puchheim, Germany). The microscope was equipped with filter sets to allow detection of the fluorescent conjugates Cy3, Alexa 488 and DAPI. For each staining and time point we took microphotographs in series with the same exposure times using MetaMorph 5.0.5 software (Molecular Devices Inc., Downingtown, PA, USA). We combined individual images to RGB color images using MetaMorph 5.0.5 software and optimized contrast and brightness of the images using Adobe Photoshop CC 2015 (Adobe Systems Incorporated, San Jose, CA, USA). Staining was performed for all animals at the same time and all images were processed the same way.

2.9. Real-time PCR

We extracted total RNA from the collected hypothalamic sections (~50 mg of tissue) using TRIzol (Invitrogen, CA, USA) according to

Table 1
Antibodies used for immunohistochemistry.

Antibody	Dilution	Catalog number
Primary antibodies		
NF-IL6 rabbit polyclonal IgG	1:6000	sc-150-G Santa Cruz Biotechnology, Santa Cruz, CA
VWF rabbit polyclonal IgG	1:5000	008202 Dako Deutschland, GmbH, Hamburg, Germany
VWF sheep polyclonal IgG	1:2000	SARF-WAG Affinity Biologicals, Ancaster, Ontario, Canada
COX-2 goat polyclonal IgG	1:2000	sc-1747 Santa Cruz Biotechnology, Santa Cruz, CA
STAT-3 rabbit polyclonal IgG	1:3000	sc-4612 Santa Cruz Biotechnology, Santa Cruz, CA
NF- κ B rabbit polyclonal IgG	1:500	sc-109 Santa Cruz Biotechnology, Santa Cruz, CA
Secondary antibodies		
Cy3-conjugated anti-rabbit IgG	1:600	711-165-152 Jackson Immuno Research West Grove, PA
Cy3-conjugated anti-goat IgG	1:600	705-165-147 Jackson Immuno Research West Grove, PA
Alexa 488-conjugated anti-rabbit IgG	1:500	A2A11055 Molecular Probes, Göttingen, Germany
Alexa 488-conjugated anti-sheep IgG	1:500	A2A11015 Molecular Probes, Göttingen, Germany

VWF = von Willebrand factor, NF-IL6 = nuclear factor for IL-6, NF- κ B = nuclear factor kappa-light-chain-enhancer of activated B cells, IgG = immunoglobulin G, STAT-3 = signal transducer and activator of transcription-3.

the manufacturer's instructions. Using 50 U murine leukemia virus (MULV) reverse transcriptase, 50 mM random hexamers and 10 mM dNTP mix (Applied Biosystems, Foster City, CA, USA) we transcribed 1 µg of total RNA into DNA; the total reaction volume was 10 µL. After reverse transcription we performed quantitative real-time PCR in duplicate using a pre-optimized primer/probe mixture (Taqman Gene Expression Assay; Applied Biosystems) and Taqman universal PCR Master Mix (Applied Biosystems) using a StepOne Real-Time PCR System (Applied Biosystems) (Damm et al., 2011). We normalized the cDNA quantities between different reactions by measuring the housekeeping gene β -actin (Applied Biosystems) as a reference. The ΔC_t mean was calculated by subtracting the C_t value for the housekeeping gene β -actin from the C_t value of the target gene. The $\Delta\Delta C_t$ was then calculated by subtracting the ΔC_t mean for each sample from a control sample within the same experiment. The control sample was the sample with the max ΔC_t mean, i.e. the lowest amount of the target gene. The RQ was then calculated as $2^{-\Delta\Delta C_t}$. The control sample therefore had an RQ of 1 and all other samples values represent an x-fold difference to the control sample (Damm et al., 2011). The assay IDs for the analyzed genes are as follows: NF-IL6 (Rn00824635_s1), ICBX (Rn01473658_g1), SOCS-3 (Rn00585674_s1), COX-2 (Rn00568225_m1), mPGES (Rn00572047_m1), TNF- α (Rn99999017_m1), IL-1 β (Rn00580432_m1), and IL-6 (Rn01410330_m1).

2.10. Experimental procedures

2.10.1. Experiment 1

Rats were assigned randomly to receive one of three different doses of 20% (w/v) brewer's yeast solution (20 ml/kg = 4 g/kg ($n = 9$), 10 ml/kg = 2 g/kg ($n = 5$) or 2 ml/kg = 0.4 g/kg ($n = 6$)) or saline (20 ml/kg of 0.9%) ($n = 6$). We administered the injections at 16:00, two hours before lights off at 18:00. For three days before and six days after the s.c. injection we monitored core body temperature, cage activity, food intake and body mass of the rats.

2.10.2. Experiment 2

A separate group of rats to those used in Experiment 1 were assigned randomly to receive one of two doses of 20% (w/v) brewer's yeast solution (20 ml/kg = 4 g/kg) or 10 ml/kg = 2 g/kg) or saline (20 ml/kg of 0.9%), before blood and brain tissue was collected under terminal anesthesia induced with an intraperitoneal (i.p.) injection of 1 ml sodium pentobarbital (Euthanase, 200 mg/ml; Kyrion Laboratories (Pty) Ltd., South Africa). We collected the blood and brain samples at 2, 8, 18 and 72 h after injection; five to seven rats were euthanized per time point for each of the intervention groups. The time points we chose corresponded to different phases of the febrile response: the early phase (2 h), the peak (8 h), the plateau phase (18 h) and the defervescence (72 h). We collected blood by cardiac puncture into sterile ethylene diamine tetraacetic acid (EDTA) tubes and then perfused the rats transcardially with ice-cold saline (0.9%) for 2 min. The blood was centrifuged at 3913 $\times g$ for 10 min and the blood plasma was stored at -80°C until processing. The whole brains we recovered were frozen on powdered dry ice for ~ 2 min and stored at -80°C until processing.

2.11. Data analysis

All data are expressed as mean and standard deviation (SD) and were analyzed using GraphPad Prism (version 7.0, GraphPad Software Inc, USA). Significance was set at $P < 0.05$. For statistical purposes, the original 5-min temperature recordings were averaged over successive two-hour periods. Abdominal temperature responses were plotted as abdominal temperature-time curves in one-hour intervals. The 5-min nocturnal activity counts for each

rat were summed for 12 h (18:00–06:00) and the summed values were averaged for three nights before the day of injection, when the rats were undisturbed in their home cages. Change in nocturnal cage activity was calculated as the difference between the night-time activity following injection and the average night-time activity in the three nights before injection and expressed as a percentage of the average pre-injection activity. Food intake was calculated as grams of food consumed in 24 h per 100 g of body mass and expressed as a percentage change from the average daily food intake measured over three days before injection. Twenty-four-hour change in body mass (g) was determined by subtracting the body mass measured at 16:00 on each day after the injection from the body mass measured immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass. The two-hourly temperature means and changes in nocturnal cage activity, food intake and body mass were analyzed by two-way repeated measures analysis of variance (ANOVA) with intervention and time as main effects. We used a Tukey post hoc test to identify differences between groups when the ANOVA detected significant main effects or interactions. To determine whether the change in cage activity, food intake and body mass on each of the six days after injection was significantly different from pre-injection values, a one-sample t -test was performed for each of the experimental groups. Cytokine levels and relative mRNA expression were compared by two-way ANOVA with treatment and time as main effects. We used a Tukey post hoc test to identify differences between groups when the ANOVA detected significant main effects or interactions. Where cytokine levels were undetectable, samples were assigned a value equivalent to the detection limit of the assay. Plasma cytokine data that were not normally distributed were log-transformed before statistical analysis. The data presented are non-transformed means and SD, but the associated significance values were derived from log-transformed data.

3. Results

3.1. Experiment 1 – Brewer's yeast induces fever, lethargy, anorexia and body mass stunting

3.1.1. Body temperature and activity

Fig. 1 shows the core body temperature over six days for rats that received s.c. injections of brewer's yeast or saline. The main effects of time ($F_{(7,2,1584)} = 121.10$, $P < 0.0001$), treatment ($F_{(3,22)} = 54.02$, $P < 0.0001$) and interaction ($F_{(21,6,1584)} = 13.83$, $P < 0.0001$) were significant such that rats injected with brewer's yeast developed a fever while rats injected with saline did not. The core body temperature of rats injected with the high dose of brewer's yeast (4 g/kg) initially decreased to $36.44 \pm 0.60^\circ\text{C}$. The decrease in core body temperature lasted for 3 h and was followed by a fever, which peaked at $39.17 \pm 0.23^\circ\text{C}$ ~ 8 h after injection. Core body temperature of rats injected with the medium (2 g/kg) or low (0.4 g/kg) doses of brewer's yeast started to increase after a latent period of ~ 3 h and peaked at ~ 8 h after injection at $39.16 \pm 0.20^\circ\text{C}$ and $38.66 \pm 0.30^\circ\text{C}$ respectively. The duration of the fever was dependent on the dose of yeast injected. Fever induced by the 0.4 g/kg dose of yeast persisted for the first night and day after injection ($P < 0.05$, Tukey), while fever induced by the medium dose (2 g/kg) and high dose (4 g/kg) persisted for five ($P < 0.05$, Tukey) and six ($P < 0.05$, Tukey) days respectively, including the first two nights.

Fig. 2 shows that s.c. injection of brewer's yeast and saline both induced a significant decrease in nocturnal cage activity, but the decrease following brewer's yeast injection was significantly greater, on the first night after injection, than that following the

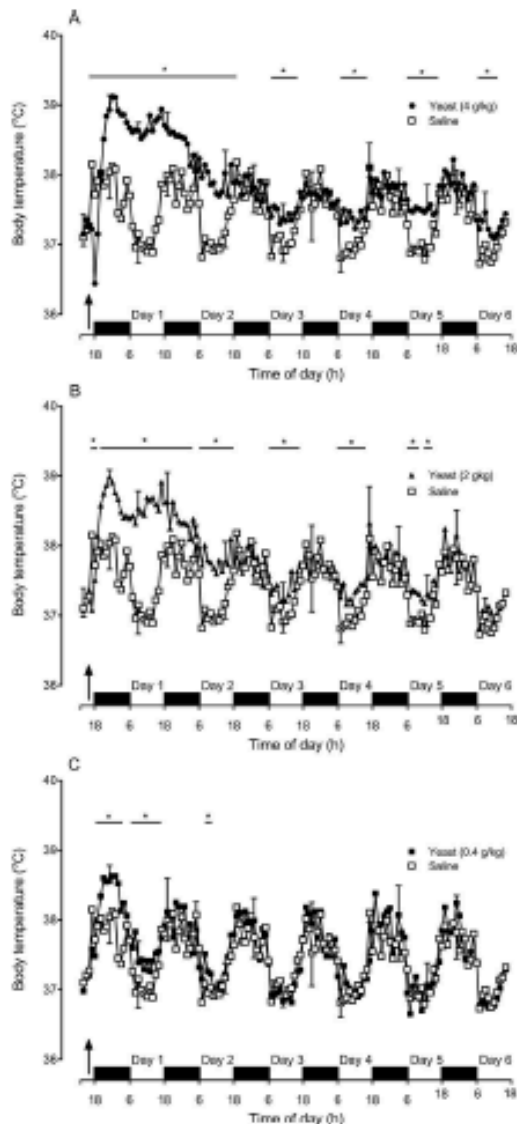


Fig. 1. Core body temperature (mean and SD) of rats over six days after receiving a subcutaneous injection of a 20% (w/v) brewer's yeast solution at a volume of (A) 20 ml/kg (4 g/kg; $n=9$), (B) 10 ml/kg (2 g/kg; $n=5$) and (C) 2 ml/kg (0.4 g/kg; $n=6$) or 20 ml/kg of 0.9% saline ($n=6$). Core body temperatures were plotted in one-hour intervals. The black arrow indicates injection time and black bars indicate lights off (18:00–06:00 clock time). *Significantly different from saline ($P < 0.05$, Tukey).

saline injection, and lasted longer (main effects of time ($F_{(5, 110)} = 54.63$, $P < 0.0001$), treatment ($F_{(2, 22)} = 74.76$, $P < 0.0001$) and interaction ($F_{(15, 110)} = 7.53$, $P < 0.0001$). Rats receiving the high and medium dose of brewer's yeast decreased nocturnal cage activity by ~80% on the first night after injection. From the third night onwards rats injected with the high or medium dose of brewer's yeast gradually became more active, but activity never fully recovered to pre-injection levels, as by the end of the sixth

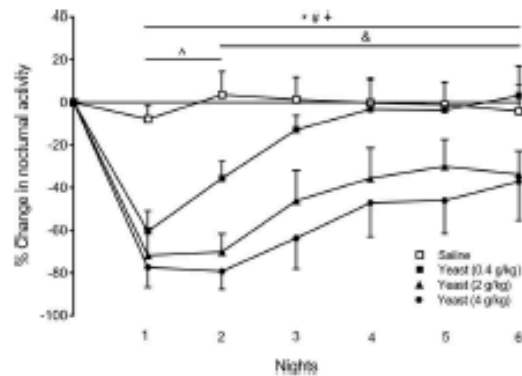


Fig. 2. Change in nocturnal activity (mean and SD) of rats over six nights after receiving a subcutaneous injection of a 20% (w/v) brewer's yeast solution at 20 ml/kg (4 g/kg; $n=9$), 10 ml/kg (2 g/kg; $n=5$) or 2 ml/kg (0.4 g/kg; $n=6$), or 20 ml/kg of 0.9% saline ($n=6$). *Indicates differences between 4 g/kg brewer's yeast and saline ($P < 0.0001$, Tukey); *Indicates differences between 2 g/kg brewer's yeast and saline ($P < 0.001$, Tukey); *Indicates differences between 0.4 g/kg brewer's yeast and saline ($P < 0.0001$, Tukey); *Indicates differences between 4 g/kg and 0.4 g/kg brewer's yeast ($P < 0.05$, Tukey); *Indicates differences between 2 g/kg and 0.4 g/kg brewer's yeast ($P < 0.01$, Tukey). One sample t test performed on each experimental group at each time point indicated that the decrease in cage activity: (1) never returned to pre-injection values for rats that received 4 g/kg or 2 g/kg brewer's yeast; (2) returned to pre-injection values on night 4 post injection for rats injected with 0.4 g/kg brewer's yeast; and (3) for rats injected with saline was only reduced compared to pre-injection values on the first night after injection.

night they remained less active than before the injections ($t = 6.1$, $P = 0.0003$ high dose; $t = 6.9$, $P = 0.002$ medium dose). Rats receiving a s.c. injection of the low dose of brewer's yeast decreased nocturnal cage activity by ~60% on the first night after injection, which was significantly less than the decrease in nocturnal activity for rats that received the high dose of brewer's yeast ($P < 0.05$, Tukey). Unlike that noted for the high and medium doses of brewer's yeast, activity recovered, from its initial decrease, such that, from the fourth night after injection rats that received the low dose of brewer's yeast had nocturnal activity levels similar to those before the injections ($t = 0.57$, $P = 0.59$).

3.1.2. Food intake and body mass

For the three days before receiving an injection the average daily food consumption of the rats per 100 g of body mass was 1.0 ± 1.1 g. The average body mass of the rats before the injection was 297 ± 13 g. Fig. 3A shows that s.c. injection of brewer's yeast induced a significant dose-dependent decrease in the food intake compared to rats injected with saline (main effects of time ($F_{(5, 110)} = 60.70$, $P < 0.0001$), treatment ($F_{(2, 22)} = 56.93$, $P < 0.0001$) and interaction ($F_{(15, 110)} = 1.5$, $P < 0.0001$)). Rats receiving the high, medium or low dose of brewer's yeast decreased food intake by approximately 60%, 50% and 20%, respectively, on the first night after injection. Food intake recovered after its initial decrease for rats injected with the high dose of brewer's yeast, but remained slightly (~8%) reduced on the sixth day after injection ($t = 2.5$, $P = 0.04$). Rats receiving the medium or lower dose of brewer's yeast had a faster recovery in food intake after the initial decrease, such that, from the fourth day after injection, they were consuming similar amounts of food compared to before the injections ($t = 2.5$, $P = 0.07$ medium dose; $t = 0.16$, $P = 0.9$ low dose).

Fig. 3B shows that the dose-dependent decrease in food intake following s.c. injection of brewer's yeast was associated with a dose-dependent loss of body mass (main effects of time ($F_{(5, 110)} = 18.19$, $P < 0.0001$), treatment ($F_{(2, 22)} = 24.95$, $P < 0.0001$)).

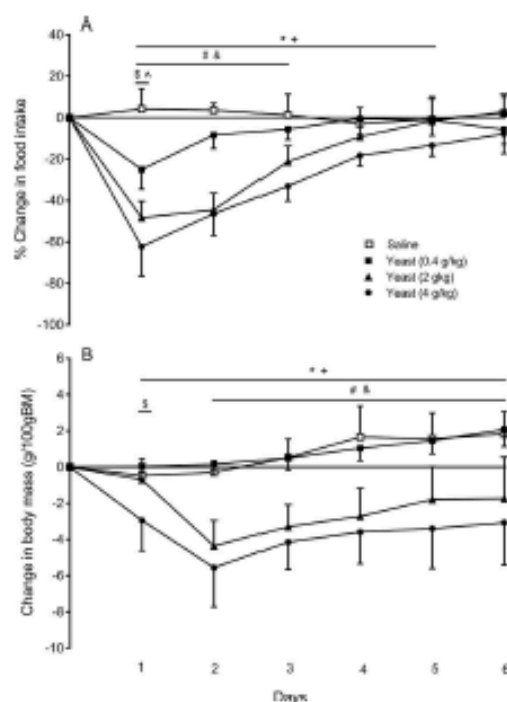


Fig. 3. Change in food intake (A) and body mass (B) (mean and SD) of rats over six days after receiving a subcutaneous injection of a 20% (w/v) brewer's yeast solution at 20 ml/kg (4 g/kg; $n = 5$), 10 ml/kg (2 g/kg; $n = 5$) or 2 ml/kg (0.4 g/kg; $n = 6$), or 20 ml/kg of 0.9% saline ($n = 6$). For the three days before receiving an injection the average daily food consumption of the rats per 100g of body mass was 10 ± 1 g. The average body mass of the rats before the injection was 297 ± 13 g. Food intake was calculated as grams of food consumed in 24 h/100g of body mass and was expressed as a percentage change from average daily food intake measured over three days before injection. Change in body mass was determined by subtracting the body mass measured on each day after the injection from the body mass measured at the same time immediately before the injection and expressed as the change in body mass (g) per 100 g of rat body mass. *Indicates differences between 4 g/kg and saline ($P < 0.05$, Tukey); * indicates differences between 2 g/kg brewer's yeast and saline ($P < 0.01$, Tukey); * indicates differences between 0.4 g/kg brewer's yeast and saline ($P < 0.0001$, Tukey); * indicates differences between 4 g/kg and 2 g/kg brewer's yeast ($P < 0.05$, Tukey); * indicates differences between 4 g/kg and 0.4 g/kg brewer's yeast ($P < 0.05$, Tukey); * indicates differences between 2 g/kg and 0.4 g/kg brewer's yeast ($P < 0.05$, Tukey). One sample t test performed on each experimental group at each time point indicated that the decrease in food intake: (1) never returned to pre-injection values for rats that received 4 g/kg brewer's yeast; (2) returned to pre-injection values on night 4 post injection for rats injected with 2 g/kg or 0.4 g/kg brewer's yeast.

and interaction ($F_{(15, 110)} = 3.45$, $P < 0.0001$). The body mass of rats injected with the high or medium dose of brewer's yeast fell progressively for two days and then gradually increased, but remained stunted on the sixth day after injection ($P < 0.01$, Tukey). Over the entire six-day period following injection, rats injected with the low dose of brewer's yeast gained a similar amount of body mass to that noted for rats injected with saline ($P > 0.05$, Tukey).

3.2. Experiment 2 – Brewer's yeast induces an inflammatory response in the periphery and the brain

3.2.1. Blood plasma levels of pro-inflammatory cytokines

We collected plasma at 2, 8, 18 and 72 h after s.c. administration of brewer's yeast or saline. Fig. 4A shows that s.c. injection

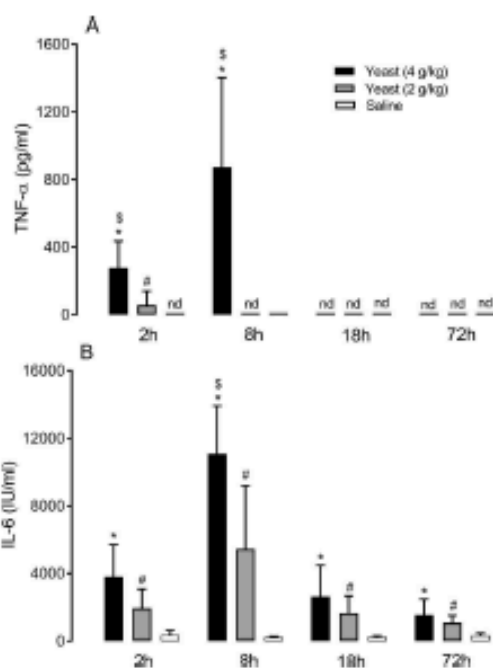


Fig. 4. Blood plasma levels (mean and SD) of bioactive TNF-α (A) and IL-6 (B) at 2, 8, 18 and 72 h after subcutaneous injection of a 20% (w/v) brewer's yeast solution at 20 ml/kg (4 g/kg); or 10 ml/kg (2 g/kg) or 20 ml/kg of 0.9% saline. Sample size per intervention group per time point = 5–7 rats. *Indicates differences between 4 g/kg brewer's yeast and saline ($P < 0.01$, Tukey); * indicates differences between 2 g/kg brewer's yeast and saline ($P < 0.05$, Tukey); * indicates differences between 4 g/kg and 2 g/kg brewer's yeast ($P < 0.05$, Tukey); nd = not detectable.

of brewer's yeast induced a significant dose-dependent increase in plasma levels of TNF-α compared to rats injected with saline (main effects of time ($F_{(2, 57)} = 46.23$, $P < 0.0001$), treatment ($F_{(2, 57)} = 98.67$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 36.93$, $P < 0.0001$)). Rats injected with the high dose (4 g/kg) of brewer's yeast had significantly elevated plasma levels of TNF-α at 2 h and 8 h compared to rats injected with the medium dose (2 g/kg) or saline ($P < 0.0001$, Tukey). Plasma levels of TNF-α were significantly elevated at 2 h in rats injected with the medium dose (2 g/kg) of brewer's yeast compared to rats injected with saline. Fig. 4B shows that s.c. injection of brewer's yeast induced a significant dose-dependent increase in plasma levels of IL-6 compared to rats injected with saline (main effects of time ($F_{(2, 57)} = 6.60$, $P = 0.0007$), treatment ($F_{(2, 57)} = 77$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 2.92$, $P = 0.0148$)). Plasma levels of IL-6 were significantly increased at all time points (2, 8, 18 and 72 h) in rats injected with the medium (2 g/kg) and high (4 g/kg) doses of brewer's yeast when compared to rats injected with saline ($P < 0.05$, Tukey). At 8 h after injection, rats that received the high dose of brewer's yeast had significantly greater levels of plasma IL-6 than rats that received the medium dose of brewer's yeast ($P = 0.04$, Tukey).

3.3. Activation of NF-IL6, STAT-3, NF-κB and COX-2 in the brain

Fig. 5 shows representative photomicrographs of immunohistochemical detection of nuclear factor interleukin-6 (NF-IL6) in the OVLT of rats injected s.c. with the high dose (4 g/kg) of brewer's

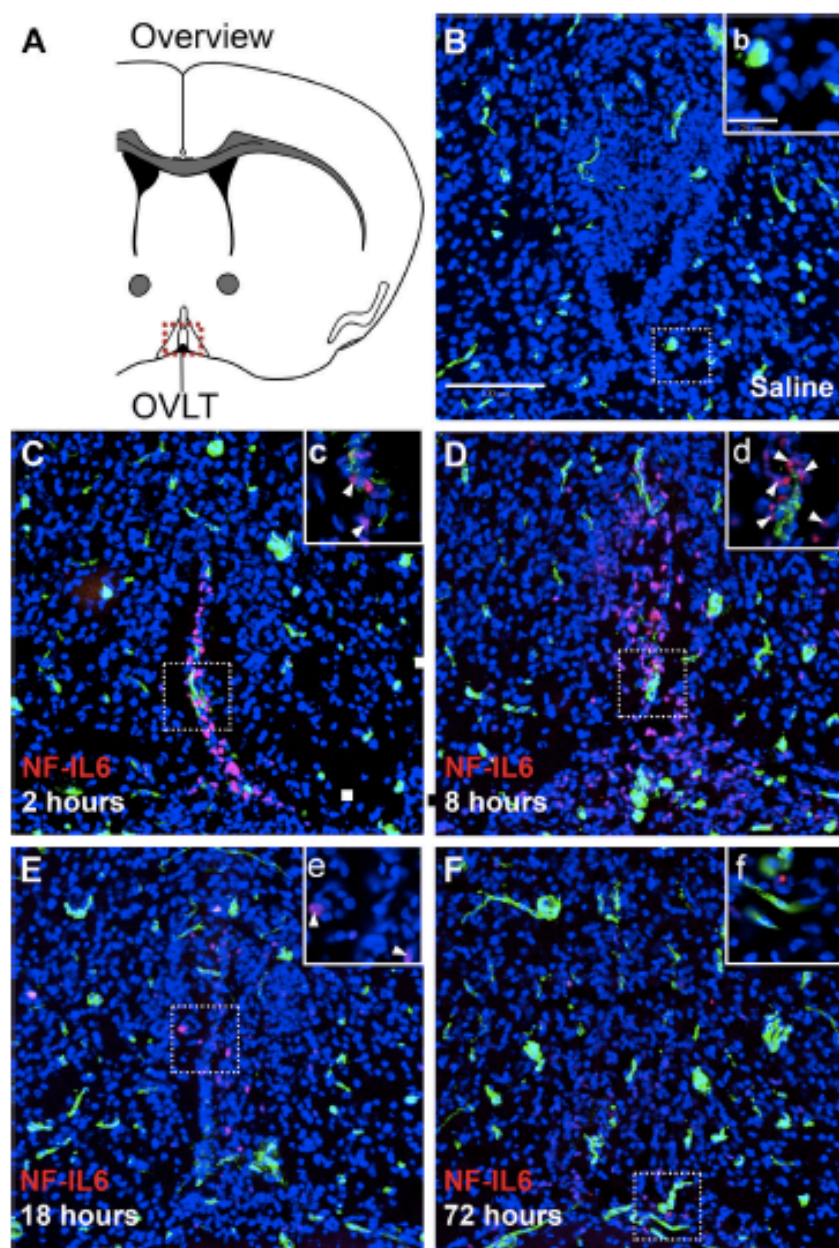


Fig. 5. Nuclear NF-IL6 immunoreactivity in the OVLT at 2, 8, 18 and 72 h after subcutaneous injection of 20 ml/kg of a 20% (w/v) (4 g/kg) brewer's yeast solution or an equal volume of 0.9% saline. (A) Schematic overview of the rat brain showing the position of the OVLT. The white arrowheads show the co-localization of red NF-IL6 signals with the nuclear DAPI stain (blue) that indicates the nuclear localization of the signals, which appeared at 2 h (C), peaked at 8 h (D) and decreased at 18 h (E) and reached basal levels at 72 h (F) after injection with brewer's yeast. Subcutaneous injection of rats with saline was not associated with nuclear NF-IL6 immunoreactivity (B). The locations of areas used for high magnification are indicated by a dashed square in the overviews. Blood vessels were stained with von Willebrand factor (green). Scale bars: in B represents 100 μ m and applies to 20 $\times$ magnification micrographs (B–E); in b represents 25 μ m and applies to 40 $\times$ magnification insets (b–e). For references to color in this figure legend, the reader is referred to the web version of this article.

yeast or saline. NF- κ B immunoreactivity, which colocalized with the nuclear DAPI stain, appeared in this sensory circumventricular organ between 2 h and 18 h after rats were injected with brewer's yeast. Rats injected with saline did not show nuclear NF- κ B immunoreactivity in the OVLT at any time points. The nuclear NF- κ B signals were closely associated with blood vessels in the OVLT as shown by an additional colocalization with von Willebrand factor (vWF). Nuclear NF- κ B immunoreactivity was also observed in blood vessels and parenchymal cells of the cortex, ependymal cells, the choroid plexus and the median eminence of rats injected with brewer's yeast (images not shown).

Fig. 6A and B shows representative high magnification photomicrographs of immunohistochemical detection of STAT-3 activation in the OVLT of rats injected with the high dose (4 g/kg) of brewer's yeast or saline. The STAT-3 immunoreactivity that colocalized with the nuclear DAPI stain, appeared in the OVLT at 2 h after injection and could still be detected at 18 h, but not 72 h, after rats were injected with brewer's yeast. Rats injected with saline did not show

nuclear STAT-3 immunoreactivity in the OVLT at any of the time points. The nuclear STAT-3 signals were closely associated with blood vessels in the OVLT, as shown by an additional colocalization with vWF. Nuclear STAT-3 immunoreactivity was also observed in blood vessels of the cortex (images not shown).

Fig. 6C and 6D shows representative high magnification photomicrographs of immunohistochemical detection of NF- κ B in the cerebral cortex of rats injected with the high dose (4 g/kg) of brewer's yeast or saline. The NF- κ B staining, which colocalized with the nuclear DAPI stain, appeared in the cortex at 2 h after injection, but was not detected in rats at 8, 18 and 72 h after injection of brewer's yeast. The nuclear NF- κ B signals were closely associated with blood vessels in the cortex as shown by an additional colocalization with vWF. Interestingly, no nuclear NF- κ B signals were observed in the OVLT at any of the time points in rats injected with brewer's yeast.

Fig. 7 shows representative high magnification photomicrographs of immunohistochemical detection of the enzyme COX-2 in

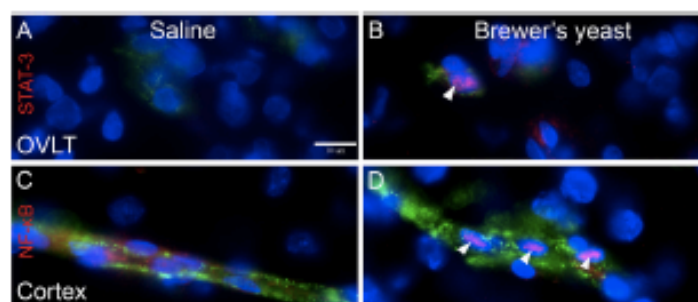


Fig. 6. Nuclear STAT-3 immunoreactivity in the OVLT (B) (red shows with white arrowheads) and nuclear NF- κ B immunoreactivity in the cerebral cortex (D) (red shows with white arrowheads) of rats 2 h after a subcutaneous injection with 20 ml/kg of a 20% (w/v) (4 g/kg) brewer's yeast solution or an equal volume of 0.9% saline. Co-localization of red NF- κ B signals with the nuclear DAPI stain (blue) indicates the nuclear localization of the STAT-3 and NF- κ B signals (red). Blood vessels were stained with von Willebrand factor (green). The nuclear STAT-3 and NF- κ B staining was associated with blood vessels, as shown by co-localization with von Willebrand factor (green). Subcutaneous injection of rats with saline was not associated with nuclear STAT-3 (A) or NF- κ B (C) immunoreactivity. Scale bar = 10 μ m and applies to all micrographs (A–D) which were taken at 100 $\times$ magnification. For references to color in this figure legend, the reader is referred to the web version of this article.

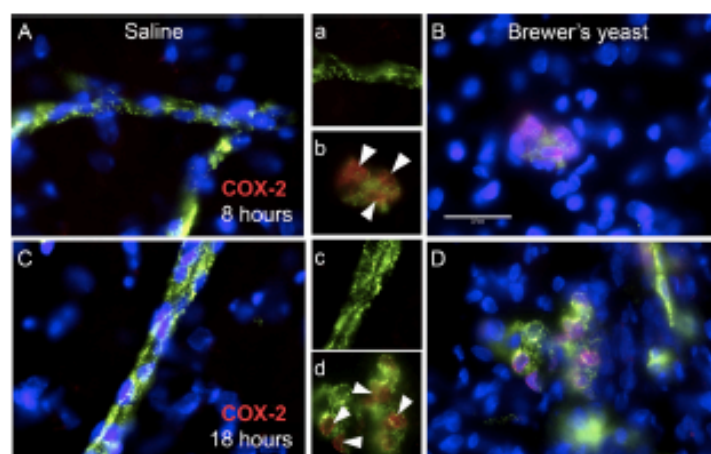


Fig. 7. Perinuclear COX-2 immunoreactivity (red) in the OVLT at 8 h (B) and 18 h (D) after subcutaneous injection of 20 ml/kg of a 20% (w/v) (4 g/kg) brewer's yeast solution or an equal volume of 0.9% saline (A and C). Co-localization of red COX-2 signals with the nuclear DAPI stain (blue) indicates the perinuclear localization of the COX-2 signals (red). COX-2 staining (red) was associated with blood vessels as shown by co-localization with von Willebrand factor (green) (white arrowheads in inserts b and d). Subcutaneous injection of rats with saline was not associated with COX-2 immunoreactivity (A) and (C) (also see inserts a and c). Scale bar = 25 μ m and applies to all micrographs (A–D) which were taken at 100 $\times$ magnification. For references to color in this figure legend, the reader is referred to the web version of this article.

the OVLT of rats injected with the high dose (4 g/kg) of brewer's yeast or saline. Typical perinuclear COX-2 immunoreactivity was detected at 8 h and 18 h, but not at 72 h after brewer's yeast injection. The perinuclear COX-2 signals co-localized with small and large blood vessels in the OVLT as shown by an additional colocalization with WF (Fig. 7B and D). Perinuclear COX-2 immunoreactivity was also detected in the walls of large vessels with large lumens in the subarachnoid space, lateral to the optic chiasm

(images not shown). No COX-2 signals were observed in blood vessels in the OVLT at any time point in rats injected with saline.

3.4. Relative mRNA expression of inflammatory mediators in the hypothalamus

Fig. 8 shows the increase in hypothalamic mRNA expression of pro-inflammatory target genes and regulatory proteins

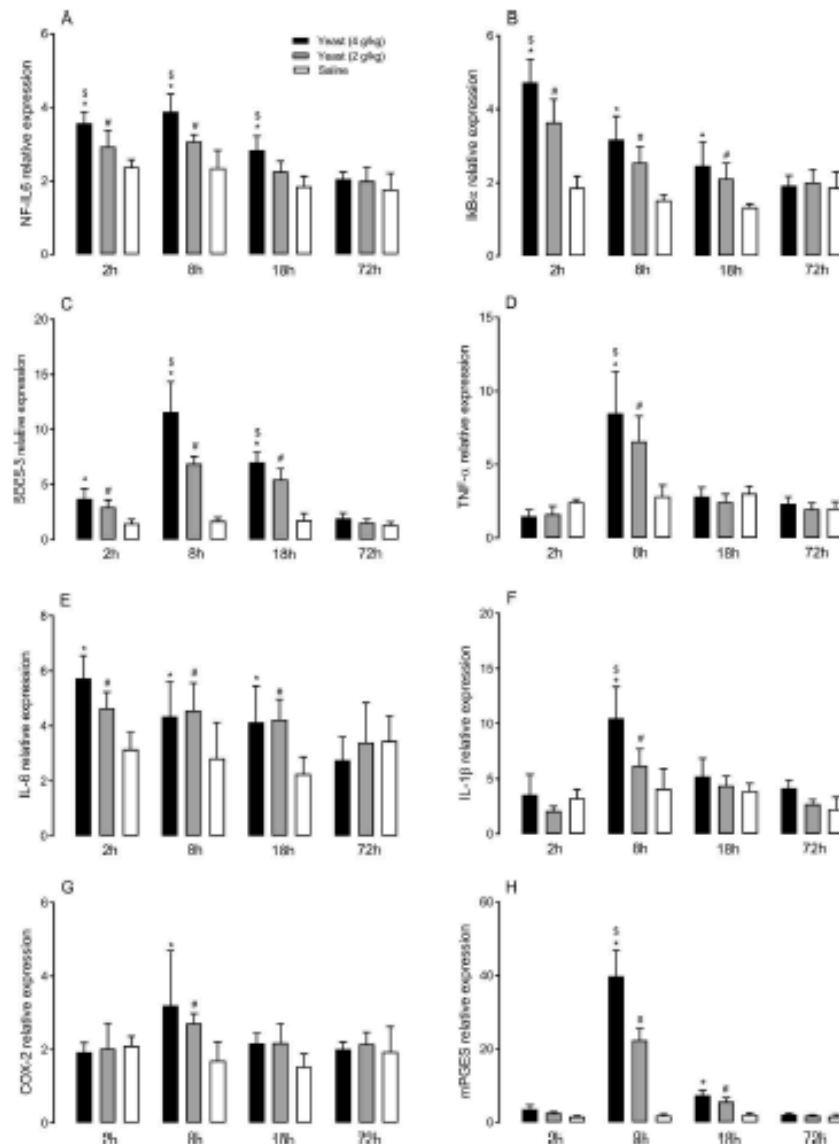


Fig. 8. Relative mRNA expression (mean and SD) of transcription factors (A–C), pro-inflammatory cytokines (D–F) and rate-limiting enzymes for PGS_2 synthesis (G–H) in the rat hypothalamus at 2, 8, 18 and 72 h after subcutaneous injection of a 20% (w/v) brewer's yeast solution at either 20 ml/kg (4 g/kg) or 10 ml/kg (2 g/kg) or 20 ml/kg of 0.9% saline. Sample size per intervention group per time point = 5–7 rats. \$ indicates differences between 4 g/kg and saline ($P < 0.05$, Tukey); * indicates differences between 2 g/kg and saline ($P < 0.05$, Tukey); # indicates differences between 4 g/kg and 2 g/kg ($P < 0.05$, Tukey).

of transcription factors following brewer's yeast injection. There was a dose-dependent increase in NF-IL6 mRNA expression in the hypothalamus of rats injected with brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (Fig. 8A; main effects of time ($F_{(2, 57)} = 40.40$, $P < 0.0001$), treatment ($F_{(2, 57)} = 46.26$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 3.07$, $P = 0.0114$). Rats injected with the high dose of brewer's yeast had significantly greater hypothalamic NF-IL6 mRNA expression at 2, 8 and 18 h compared to rats injected with the medium dose or saline ($P < 0.05$, Tukey). The upregulation of NF-IL6 mRNA in the hypothalamus was significantly greater in rats injected with the medium dose compared to rats injected with saline at 2 and 8 h ($P < 0.05$, Tukey).

Fig. 8B shows that there was a dose-dependent increase in the mRNA expression of the NF- κ B regulator I κ B α in the hypothalamus of rats injected with brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(2, 57)} = 38.55$, $P < 0.0001$), treatment ($F_{(2, 57)} = 54.62$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 8.84$, $P < 0.0001$). Rats injected with the high or medium dose of brewer's yeast had significantly greater hypothalamic I κ B α mRNA expression at 2, 8 and 18 h compared to rats injected with the saline ($P < 0.05$, Tukey). The upregulation of I κ B α mRNA in the hypothalamus was significantly greater in rats injected with the high dose of brewer's yeast compared to rats injected with medium dose of brewer's yeast at 2 h ($P < 0.001$, Tukey).

Fig. 8C shows that there was a dose-dependent increase in SOCS-3 mRNA expression in the hypothalamus of rats injected with brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(2, 57)} = 80.61$, $P < 0.0001$), treatment ($F_{(2, 57)} = 109.10$, $P < 0.0001$) and their interaction ($F_{(6, 57)} = 21.51$, $P < 0.0001$). Rats injected with the high or medium dose of brewer's yeast had significantly greater hypothalamic SOCS-3 mRNA expression at 2, 8 and 18 h compared to rats injected with the saline ($P < 0.05$, Tukey). The upregulation of SOCS-3 mRNA in the hypothalamus was significantly greater in rats injected with the high dose of brewer's yeast compared to rats injected with medium dose of brewer's yeast at 8 and 18 h ($P < 0.05$, Tukey).

Fig. 8D and E shows that there was a dose-dependent increase in TNF- α and IL-1 β mRNA expression in the hypothalamus of rats injected with brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (TNF- α : main effects of time ($F_{(2, 57)} = 51.94$, $P < 0.0001$), treatment ($F_{(2, 57)} = 6.82$, $P = 0.0022$) and interaction ($F_{(6, 57)} = 11.19$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 21.51$, $P < 0.0001$); IL-1 β : main effects of time ($F_{(2, 57)} = 28.90$, $P < 0.0001$), treatment ($F_{(2, 57)} = 19.71$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 5.31$, $P = 0.0002$). Rats injected with the high or medium dose of brewer's yeast had significantly greater hypothalamic TNF- α and IL-1 β mRNA expression at 8 h compared to rats injected with the saline ($P < 0.05$, Tukey). The upregulation of TNF- α and IL-1 β mRNA in the hypothalamus was significantly greater in rats injected with the high dose of brewer's yeast compared to rats injected with medium dose of brewer's yeast at 8 h ($P < 0.05$, Tukey).

Fig. 8E shows that there was an increase in IL-6 mRNA expression in the hypothalamus of rats injected with brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(2, 57)} = 5.34$, $P = 0.0026$), treatment ($F_{(2, 57)} = 12.51$, $P < 0.0001$) and interaction ($F_{(6, 57)} = 2.99$, $P = 0.0132$). Rats injected with the high or medium dose of brewer's yeast had significantly greater hypothalamic IL-6 mRNA expression at 2, 8 and 18 h compared to rats injected with saline ($P < 0.05$, Tukey). There were no significant differences in the upregulation of IL-6 mRNA in the hypothalamus between rats injected with the high dose of brewer's yeast compared to rats injected with medium dose of brewer's yeast ($P > 0.05$, Tukey).

Fig. 8G shows that there was an increase in COX-2 mRNA expression in the hypothalamus of rats injected with brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main

effects of time ($F_{(2, 57)} = 3.46$, $P = 0.0221$), treatment ($F_{(2, 57)} = 4.87$, $P = 0.0111$) and their interaction ($F_{(6, 57)} = 2.28$, $P = 0.0485$). Rats injected with the high or medium dose of brewer's yeast had significantly greater hypothalamic COX-2 mRNA expression at 8 h compared to rats injected with saline ($P < 0.05$, Tukey). There were no significant differences in the upregulation of COX-2 mRNA in the hypothalamus between rats injected with the high dose of brewer's yeast compared to rats injected with medium dose of brewer's yeast ($P > 0.05$, Tukey).

Fig. 8H shows that there was a dose-dependent increase in mPGES mRNA expression in the hypothalamus of rats injected with brewer's yeast (4 g/kg or 2 g/kg) compared to rats injected with saline (main effects of time ($F_{(2, 57)} = 250.40$, $P < 0.0001$), treatment ($F_{(2, 57)} = 130.40$, $P < 0.0001$) and their interaction ($F_{(6, 57)} = 76.69$, $P < 0.0001$). Rats injected with the high or medium dose of brewer's yeast had significantly greater hypothalamic mPGES mRNA expression at 8 and 18 h compared to rats injected with saline ($P < 0.05$, Tukey). The upregulation of mPGES mRNA in the hypothalamus was significantly greater in rats injected with the high dose of brewer's yeast compared to rats injected with medium dose of brewer's yeast at 8 h ($P < 0.0001$, Tukey).

4. Discussion

We have shown that subcutaneous (s.c.) administration of brewer's yeast dose-dependently induces fever, lethargy, anorexia and body mass stunting that is accompanied by inflammation in the periphery and the brain. To our knowledge this is the first report that fever and sickness behaviors induced by brewer's yeast administration coincides with increased concentrations of circulating pro-inflammatory cytokines (IL-6 and TNF- α) (Fig. 4), and enhanced mRNA and protein expression of key transcription factors (NF-IL6, STAT-3, NF- κ B) and their respective regulators (I κ B α and SOCS-3) involved in the activation or suppression of immune response genes in the brain (Figs. 5–8). In particular, we noted a pronounced increase in hypothalamic mRNA expression of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and the rate-limiting enzymes for prostaglandin synthesis (COX-2 and mPGES) primarily around the peak of fever, which occurred eight hours after s.c. administration of brewer's yeast (Figs. 1 and 8). Moreover, we noted nuclear immunoreactivity for NF-IL6 and STAT-3 and perinuclear COX-2 immunoreactivity that was associated with blood vessels in the OVLT of rats injected with brewer's yeast (Figs. 5–8). Thus, the OVLT appears to be an important communication pathway between the periphery and the brain during yeast-induced infections.

The fever, lethargy, anorexia and body mass stunting we observed in the rats was dose-dependent in that the high (4 g/kg) and medium (2 g/kg) doses of brewer's yeast both induced responses of greater magnitude and longer duration than did the lower dose of brewer's yeast (0.4 g/kg) (Figs. 1–3). Administering the high dose of brewer's yeast was however associated with an initial decrease in core body temperature ($\sim 0.8^\circ\text{C}$) before the onset of the fever, excessive inflammation around the head and neck region on the first night and day after injection, and the subsequent development of an abscess. These responses did not occur following administration of the medium or low dose of brewer's yeast. Thus, in terms of animal welfare the medium dose of brewer's yeast may be the more appropriate dose to use for antipyretic screening studies.

Published pharmacological antipyretic testing guidelines in rats recommend that the test compound or the standard drug be administered 18 h after injection of yeast and temperatures be recorded at intervals of 30–60 min for five hours thereafter (Thangaraj, 2016; Vogel, 2002). In our hands, where we injected

brewer's yeast (4 g/kg and 2 g/kg) approximately two hours before lights off, 18–23 h after injection corresponded to the plateau phase of the fever. During the plateau phase of the fever, abdominal temperature of rats receiving the high and medium doses of brewer's yeast was consistently greater (1–1.5 °C) than the abdominal temperature of rats that received saline. The clear and consistent increase in body temperature we noted during the plateau phase of the fever supports the recommendation of antipyretic administration taking place 18 h after brewer's yeast administration and identifies that screening for antipyretic effects can continue beyond 23 h. However, it is important to note that the fever was prominent only during the lights-on phase and masked during the lights-off phase, due to the nycthemeral temperature rhythm of rats.

In addition to determining the yeast-induced fever-curve and sickness behaviors across a range of brewer's yeast doses commonly used in antipyretic screening in rats, we also measured the inflammatory response in the periphery and brain. The time-course of the inflammatory changes showed a marked increase in the synthesis of pro-inflammatory cytokines, key prostaglandin synthesizing enzymes and inflammatory transcription factors in the periphery and brain between 2 and 18 h after brewer's yeast administration, which corresponded to the onset, peak and plateau of the fever. Overall, the most pronounced increase in inflammatory mediators was noted at the fever peak, eight hours after brewer's yeast injection, with a muted response at 18 h (Figs. 4–8). Thus, for studies wanting to explore the mechanism of action of antipyretics, they may want to consider administering the antipyretic around 8 h after brewer's yeast administration, rather than 18 h after brewer's yeast administration, as is currently recommended.

Characterizing the inflammatory response to brewer's yeast is not only beneficial for studies using brewer's yeast to screen antipyretics, but it also improves our understanding of the interaction between the immune system and fungal pathogens. The major pattern recognition receptors activated during fungal infections are Toll-like receptors (TLR2, 6 and 9), C-type lectin receptors (CLRs), galectin family proteins and receptor for advanced glycation end-products (RAGE) (Romani, 2011). Binding of specific fungal pathogen associated molecular patterns, such as β -glucans, chitin, mannans linked to proteins through N- or O-linkages and fungal nucleic acids, to TLRs and CLRs, activates numerous intracellular pathways and the synthesis of immunoregulatory proteins, such as cytokines (Romani, 2011).

The fever, lethargy, anorexia and body mass stunting induced by s.c. administration of brewer's yeast we noted in our study was accompanied by an increase in plasma levels and hypothalamic mRNA expression of the pro-inflammatory cytokines IL-6 and TNF- α (Figs. 4 and 8). A role for brain IL-6 and brain TNF- α in mediating fever associated with fungal infections has been suggested, based on the findings that intracerebroventricular (i.c.v.) injection of antibodies to IL-6 and TNF- α significantly attenuates fever induced by peripheral administration of zymosan, a cell wall extract of *S. cerevisiae* (Bastos-Pereira et al., 2014). However, with no studies to date having investigated the endogenous role of peripherally-released IL-6 and peripherally-released TNF- α in mediating fever during fungal infections, the role of circulating IL-6 and TNF- α in mediating brewer's yeast-induced fever is less clear. Despite the lack of direct evidence linking circulating IL-6 and TNF- α in mediating brewer's yeast-induced fever in our study, observations obtained from studies investigating the endogenous role of these cytokines in mediating fever induced by administration of LPS, the glycolipid pyrogenic moiety of the Gram-negative bacterial membrane, provide support for their pyrogenic capabilities. In particular, we have previously noted that neutralizing the action of IL-6 or TNF- α in the periphery, significantly attenuates

fever induced by peripheral administration of LPS (Cartmell et al., 2000; Harden et al., 2006; Rummel et al., 2006; Roth et al., 1998). Furthermore, the plasma levels of IL-6 we noted following s.c. administration of brewer's yeast were comparable to those noted following s.c. administration of LPS (Harden et al., 2011). Recent studies have shown that endogenously released IL-6 exerts its pyrogenic effect during LPS-induced systemic inflammation by binding to IL-6 receptors on brain endothelial cells (Eskilsson et al., 2014).

In addition to an increase in hypothalamic mRNA expression of pro-inflammatory cytokines, we also noted an increase in hypothalamic mRNA expression of the enzymes COX-2 and mPGES. Coupled induction of COX-2 and mPGES results in the synthesis of the critical terminal mediator of fever, PGE₂ (Saper et al., 2012; Wilhelmus et al., 2014; Rummel et al., 2011; Vasilache et al., 2015). Using an animal model of arthritis, where zymosan is injected into the knee joint, others have shown that zymosan-induced fever is attenuated by administration of COX-2 selective inhibitors and non-selective COX-inhibitors (Kanashiro et al., 2009). Moreover, the attenuated zymosan-induced fever with administration of COX-2 selective inhibitors was accompanied by a decrease in CSF PGE₂ concentration (Kanashiro et al., 2009). Thus, brain-based PGE₂ may be an important mediator of brewer's yeast-induced fever.

Not only are IL-6, TNF- α and PGE₂ likely to play a role in mediating brewer's yeast-induced fever in rats, but so too the sickness behaviors of lethargy and anorexia. Although we did not directly investigate the endogenous role of IL-6, TNF- α and PGE₂ in mediating lethargy and anorexia induced by s.c. administration of brewer's yeast, we and others have shown that endogenous antagonism of IL-6, TNF- α or PGE₂ can attenuate LPS-induced anorexia and lethargy (Kozak et al., 1995; Töllner et al., 2000; Sharma et al., 1992; Porter et al., 2000; Harden et al., 2011). In addition to endogenous antagonism of IL-6, TNF- α or PGE₂ attenuating LPS-induced anorexia and lethargy, so too does endogenous antagonism of brain IL-1 β (Harden et al., 2011; Layé et al., 2000). Our finding of increased mRNA expression of IL-1 β and PGE₂ synthesizing enzymes (COX-2 and mPGES) in the hypothalamus eight hours after injection of brewer's yeast indicates that they may also be important endogenous mediators of yeast-induced sickness behaviors. The observation made by others that administering COX inhibitors significantly attenuates zymosan-induced sickness behavior in mice further supports a likely role for prostaglandins in mediating brewer's yeast-induced sickness behaviors (Lima et al., 2016).

The synthesis of the pro-inflammatory cytokines and prostaglandins proposed to be involved in mediating fever and sickness behaviors occurs via activation of inflammatory transcription factors, such as NF-IL6, STAT-3 and NF- κ B (for review see Rummel (2016)). Following activation, transcription factors translocate from the cytoplasm into the nucleus where they regulate the expression of inflammatory target genes identified as important mediators of fever and sickness behaviors (for review see Roth and Blatteis (2014), Pecchi et al. (2009)). Using immunohistochemical staining and real-time PCR we examined the mRNA expression, activation and translocation of the transcription factors in the brains of rats injected with brewer's yeast. While the signals were observed in several structures in the brain namely the cerebral cortex, choroid plexus, ependymal cells and median eminence, our main areas of interest were the hypothalamus and the OVLT. The OVLT expresses very low basal levels of nuclear NF-IL6 and STAT-3 (Nadeau et al., 2005; Rummel et al., 2004), but activation and nuclear translocation of these transcription factors increases dramatically during inflammation induced by LPS administration (Damm et al., 2011; Rummel et al., 2004; Voss et al., 2007; Rummel et al., 2005; Gautron et al., 2002). Immunohistochemical staining for nuclear translocation and activation of transcription factors in this sensory CVO, therefore, serves as a useful tool for

investigating yeast-related inflammatory signaling between the periphery and the brain.

In our study, a clear, time-dependent activation of NF- κ B and STAT-3, but not NF- κ B was observed in the OVLT following injection of brewer's yeast (see Figs. 5 and 6). In line with the increase in immunoreactivity of NF- κ B in the OVLT, we also noted increased mRNA expression of NF- κ B in the hypothalamus. Interestingly, despite the absence of NF- κ B activation in the OVLT at the protein level, the expression of its regulatory inhibitory molecule I κ B α , which usually matches its activation patterns (Quan et al., 1997), was significantly increased in the hypothalamus and peaked at the earliest time-point of two hours. NF- κ B activation in the OVLT has been shown to occur as early as 30 min after peripheral injection of IL-1 β (Nadjar et al., 2003) and, thus, it is possible that our earliest time point (2 h) was rather late to detect its nuclear translocation in the OVLT. NF- κ B translocation was, however, observed in blood vessels of the cerebral cortex (see Fig. 6D). Nuclear STAT-3 translocation occurred at two hours and lasted until 18 h. STAT-3 activation has been implicated in the expression of SOCS-3, which mediates feedback inhibition of cytokine signaling pathways (Alexander, 2002). Hypothalamic mRNA expression of SOCS-3 was increased between two hours and 18 h after s.c. administration of brewer's yeast (Fig. 8). Overall, the cascade of cytokine release in the periphery explains the activation of inflammatory transcription factors in the brain following brewer's yeast administration. To varying degrees the activation of NF- κ B, STAT-3 and NF- κ B in the hypothalamus and OVLT appear to be involved in the initiation and maintenance, and SOCS-3 and NF- κ B in the termination of the brain inflammatory and sickness response to s.c. administration of brewer's yeast; a similar sequence has been observed during systemic inflammation induced by LPS (Damm et al., 2011; Rummel, 2016; Schneiders et al., 2015).

Our results show that the fever and accompanying sickness behaviors induced by brewer's yeast outlasted the increase in inflammatory mediators observed in the periphery and the brain. A potential explanation for the sickness responses outlasting the inflammatory mediators relate to brewer's yeast being injected s.c. between the shoulder blades and the subsequent development of inflammation around the head and neck region. By the sixth day after administration of the high dose of brewer's yeast an abscess had developed. Others have noted that brewer's yeast abscess formation can subsequently develop into a chronic granuloma (Todd, 1986). Using a model of localized inflammation in rats and guinea-pigs, we and others have identified that cutaneous afferent neural signals may participate in the transmission of febrile signals from the localized site of peripheral inflammation to parts of the brain which control fever and sickness behavior induction (for review see Roth and De Souza (2001)). Thus, the signal for the low-grade fever noted in our study between the second and sixth day after brewer's yeast administration could be due to local cytokine production at the site of inflammation around the head and neck region.

In conclusion, we have identified a marked increase in the synthesis of pro-inflammatory cytokines, key prostaglandin synthesizing enzymes and inflammatory transcription factors in the periphery and brain between 2 and 72 h after subcutaneous administration of brewer's yeast. Overall, the most pronounced increase in inflammatory mediators was noted at the fever peak, eight hours after brewer's yeast injection, with a muted response at and beyond 18 h after injection. Thus, for studies wanting to explore the mechanism of action of antipyretics, they may want to consider modifying the current recommendation of administering the antipyretic 18 h after brewer's yeast administration. Moreover, our immunohistochemical findings indicate that endothelial cells, particularly within the CVOs, are likely key sites for the

synthesis of inflammatory mediators involved in regulating sickness responses during yeast infections. The immunogenicity of *S. cerevisiae*, demonstrated in our study, is a clinically important finding in light of the ubiquity and extensive use of *S. cerevisiae* in baking, brewing, agricultural and biotechnological applications. Exposure to viable yeast cells, particularly in immunocompromised individuals, may predispose them to invasive fungal infections.

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