The Effects of Prenatal Stress on the Preoptic Nuclei of Febrile Seizure Rat Models

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TITLE PAGE

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Submitted as the dissertation component in partial fulfilment for the degree of Masters of Physiology in the school of Laboratory Medicine and Medicinal Sciences, University of Kwazulu-Natal

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DECLARATION

I, Yasmin Malik, declare as follows:

That the work described in this thesis has not been submitted to UKZN or another tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

Similar work under the name: The Effects of Prenatal Stress on the Preoptic Nuclei of a Febrile Seizure Rat Model (AREC/022/017H), was submitted by myself to UKZN for an honour's degree in medical science in 2017. Preliminary findings from my previous work indicated complications with regards to the animal model used to study febrile seizures. The full study was thus not completed, and the shortcomings of the model reported on. My previous work lead to the research for my current study to redesign the current animal model used in our laboratory, as well to introduce a new model, all while still assessing the exacerbated effects of prenatal stress on the hypothalamus on rat models of febrile seizure. A new ethics application (AREC/045/018M) was obtained for the work in this study.

That my contribution to the project was as follows:

All research theory, animal work, laboratory analysis and the compiling of data, as well as the writing of this dissertation was done by myself.

That the contributions of others to the project were as follows:

Dr Lihle Qulu, my supervisor, oversaw all my work through this project.

Student: Yasmin Malik, 217032991 Date:

Supervisor: Dr. Lihle Qulu Date:

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ACRONYMS

ACTH	adrenocorticotropic hormone
AAP	American Academy of Pediatrics
ANOVA	analysis of variance
avg.	average
B	blank
BBB	blood brain barrier
CEC	cornified squamous epithelial cells
cDNA	complementary DNA
CNS	central nervous system
COX-2	cyclooxygenase-2
Cq	quantification cycle
CRH	Corticotrophin-releasing hormone
DNA	deoxyribonucleic acid
e.g.	*exempli gratia:* for example
ELISA	enzyme-linked immunosorbent assay
EP_3R	prostaglandin EP3 receptors
FBR	Foundation for Biomedical Research
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
GND	gestational day
GR	glucocorticoid receptors
HPA	hypothalamus-pituitary-adrenal
HSD-2	hydroxysteroid-dehydrogenase 2
h	hour
i.e.	*id est:* in other words
i.p.	intraperitoneally
IL-1	interleukin 1
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IL-1RAcP	interleukin-1 receptor accessory protein
KA	kainic acid

KAR	kainate receptor
Kg	kilogram
LEU	leukocytes
LPO	lateral preoptic nuclei
LPS	lipopolysaccharide
mg	milligram
min	minute
MnPO	median preoptic nuclei
MPO	medial preoptic nuclei
mRNA	mitochondrial RNA
NHGRI	National Human Genome Research Institute
NEC	oval nucleated epithelial cells
Mn	nanometre
NS	non-stressed
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E2
PGs	prostaglandins
PND	postnatal day
POA	preoptic area
RNA	ribonucleic acid
S	stress
TLE	temporal lobe epilepsy
TNF	tumour necrosis factor
X	horizontal value in a pair of coordinates
Y	vertical value in a pair of coordinates

STUDY OUTLINE

This masters dissertation is presented in article format, with one study and four chapters.

A background has been included to orientate the reader to the overall view of the study problem, statements, as well as our aims. Chapter one is a literature review which is a general discussion based on the topics covered in the study. Chapter two contains the manuscript submitted to Journal of Neuroscience Methods. The manuscript is written in accordance to the journal guidelines. Chapter three contains the overall synthesis for the study, as well as future recommendations. Chapter four contains additional appendices.

ABSTRACT

Introduction

Febrile seizures are a neurological abnormality that occurs after an underlying systemic infection, leading to fever and followed by convulsions due to neuronal hyper-excitability. Although regarded as benign, prenatal stress, prominent in third world countries, has been shown to exacerbated febrile seizures in offspring by dysregulating the hypothalamus-pituitary-adrenal axis, subsequently leading to increased production of prostaglandin E_2 (PGE_2) in the preoptic nuclei region of the hypothalamus. Current animal models used to mimic febrile seizures include but are not limited to, lipopolysaccharide (LPS) and kainic acid (KA) administration, or exposure to a hyperthermic environment. However, these models often result in 50% seizure onset with high mortality rates. Therefore, the aims of this study were to refine the models and assess the effects of prenatal stress.

Materials and Methods

Pregnant Sprague Dawley dams were exposed to restraint stress in their third trimester, and febrile seizures induced in the subsequent pups on postnatal day 14 by one of 2 models i.e. 1. Inducing febrile seizure using the LPS and KA method and 2. via exposure to a hyperthermic environment. Seizures were scored 0 to 5 on a Racine scale. Hypothalamic tissue was harvested and assessed for PGE_2 and its receptor (EP_3R) concentrations by means of ELISA and PCR respectively.

Results

Our findings show that modifying the dose to 50 µg/kg LPS and 0.44 mg/kg KA significantly decreased mortality, and thus proved to be most effective. We were also able to induce convulsions through the hyperthermic model. Additionally, we showed that exposure to prenatal stress significantly exacerbated fever and seizure severity, and increased EP_3R concentrations.

Conclusion and Relevance

The modified LPS-KA and heat models both proved sufficient in inducing convulsions, with drastically reduced mortality rates for both stressed and non-stress rat offspring. Albeit it was slightly less severe than previously reported, these simple benign seizures more accurately mimic simple febrile seizures most often experienced by otherwise healthy infants and young children. The hyperthermic model, although sufficient in inducing seizures and maintaining survival, was unable to mimic fever though the appropriate PGE_2 release, and thus the modified LPS-KA model was selected as the most effective and most efficient model to use in order to study febrile seizures.

BACKGROUND

It is estimated that the global prevalence of febrile seizures is 3 - 5% of the population, with sub-Saharan Africa showing an alarmingly higher prevalence of 0.8 - 31% (1-3). It is the most common convulsive disorder in neurologically healthy children under the age of 6 (4). A febrile seizure is a neurological abnormality that occurs after an underlying systemic infection, leading to an elevation in core body temperature know as a fever, followed by neuronal hyper-excitability and thus convulsions (5-7). This elevated body temperature is often caused by upper respiratory tract, middle and inner ear, or gastrointestinal tract infections which trigger the onset of febrile seizures (7, 8). Febrile seizures are most often associated with general influenza (9), but are also associated with roseola virus infections such as human herpesvirus-6, as well as after some childhood immunizations (10-12). It should be noted however that the immunisations themselves do not increase the risk of febrile seizures, but rather the fever often experienced afterwards (13, 14).

The systemic immune response to the above mentioned infections mobilize the release of various proinflammatory cytokines (15) such as interleukin-1 beta, interleukin 6, and tumour-necrosis factor alpha (16). Due to the increased production and release of these proinflammatory cytokines, the blood brain barrier becomes compromised resulting in a neurotransmitter imbalance between glutamate and γ-aminobutyric acid (GABA) triggering the onset of convulsions (17, 18). Interleukin-1 beta (IL-1β), a fever promoting pyrogen (19-21), has been shown to play a pivotal role in febrile seizure development during infection (5) by activating cyclooxygenase-2 (COX-2) to catalyse the conversion of arachidonic acid into prostaglandin E_2 (PGE_2) in the preoptic nuclei (16). The preoptic nuclei, or preoptic area (POA) is found in the rostral most part of the hypothalamus (22) and is often referred to as the fever centre (6). PGE_2 acts to induce fever by inhibiting preoptic warm-sensitive neurons and increasing preoptic cold-sensitive neurons, thus suppressing heat loss and enhancing heat production and retention respectively (6, 23). This effectively elevates the hypothalamic set-point temperature, leading to fever (6, 23).

Numerous studies have been conducted in our laboratory attempting to understand factors that perpetuate febrile seizures and if neuronal malfunctions can result from these convulsions. Our findings have shown that febrile seizures resulted in neuronal developmental malformation and behavioural changes (7, 19, 20, 24). Furthermore, we have shown that exposure to prenatal stress resulted in exacerbated febrile seizures in rat offspring through increased cytokine production, subsequently leading to more pronounced malformations (7, 21). Prenatal stress accounts for all types of stress, i.e. emotional and or physical stress experienced by pregnant mothers, with the most prominent effects occurring during their last trimester of pregnancy (7, 19, 21).

A wide range of animal models have been adapted to study the effects of febrile seizures, e.g. administration of various chemoconvulsants, electrical stimulation, or exposure to heated environments (7, 19, 20, 25-27). Our laboratory adapted the Heida et al. (2005) (3) chemoconvulsants model, whereby febrile seizures are induced in 14 day old rats with a combination of lipopolysaccharide (LPS) and kainic acid (KA) to mimic infection and convulsions respectively. Heida et al. (2005) obtained febrile seizures in at least 50% of animals without recurrent seizures using 200µg/kg of LPS followed 2 h 30 min later by 1.75mg/kg of KA administered intraperitoneally (3). The model is effective in inducing febrile seizures, however it has a high mortality rate. This lead us to refine the Heida et al. (2005) (3) model, as well as to the introduce a hyperthermic model based on previous models by Jiang et al. (1999) (28) and Yagoubi et al. (2015) (29), for use in our laboratories. In these hyperthermic models, young rats ranging from 10 to 15 days old were placed in heated environments for a brief period to increase core body temperature. In each case, as soon as signs of seizures occurred, the rats were removed from the hyperthermic environment, placed on a cool surface and observed for seizure activity (29-32).

Thus, the focus of this study was to:

1. Determine the most effective and efficient dose of LPS and KA to induce febrile seizures in rats, while maintaining a high survival rate.
2. Determine the most effective and efficient LPS-KA and hyperthermia based animal models of febrile seizure to be used in our laboratory.
3. Assess the neurochemical effect of prenatal stress on PGE_2 and its receptor EP_3R concentration in hypothalamic tissue of LPS-KA and hyperthermia based animal models of febrile seizure.

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CHAPTER ONE

Chapter one contains the literature review for this manuscript. The literature review covers febrile seizure epidemiology, pathophysiology, as well as relevant cytokines that lead to fever and convulsions. The literature review also discusses various animal models used to study febrile seizures.

LITERATURE REVIEW

EPIDEMIOLOGY

The American Academy of Paediatrics (AAP) defines febrile seizures as seizures that occur during fever in the absence of a history of afebrile seizures, metabolic disturbance, intracranial infection or any other central nervous system (CNS) infections (1). Globally, febrile seizures are experienced by 2 - 5% of children between the ages of 3 months to 6 years (1-4), and 0.8 - 31% of children in sub-Saharan Africa (5), with a peak incidence around 18 months of age (6). Africa has shown a growing prevalence of acute seizures with risk factors including, poor sanitation, malnutrition, inadequate medical facilities and resources, as well as insufficient knowledge (4, 7, 8). These deficiencies leave children at higher risk of systemic infections such as middle ear infection, gastroenteritis and upper respiratory tract infections that result in fever (4, 7, 8). Febrile seizures may arise from the ensuring systemic fever occurring as a result of these above mentioned infections, thereby evoking excessive neuronal impulse firing that may result in convulsions (8, 9).

Febrile seizures can be divided into three main categories i.e. simple or complex febrile seizures, and status epilepticus (5, 6, 8). Simple febrile seizures last 1-2 minutes and have a clear focus of infection (3). Complex febrile seizures last 15-30 minutes and may reoccur within a single fever episode, while status epilepticus occurs arbitrarily in the brain during a febrile infection, lasts longer than 30 minutes, and reoccurs within a 24 h window (6, 8, 10). Complex febrile seizures and status epilepticus have been known to develop into more severe conditions such as temporal lobe epilepsy (TLE) (2, 6, 8, 9, 11, 12). A 50% increased risk for epilepsy was found in neonates that had a family history of TLE, had complex febrile seizures or those that had neurodevelopmental impairments (11). According to the National Health Service (NHS, UK, 2018) it is estimated that healthy children have a 1% chance of developing epilepsy later in life, as compared to a 2% and 5% chance for those who experience simple and complex febrile seizures respectively (13).

FEBRILE SEIZURE PATHOPHYSIOLOGY

Febrile seizures occur with the activation of the inflammatory immune response to an insult, thereby releasing activated leukocytes such as macrophages and neutrophils, which in turn release pro-inflammatory cytokines, predominantly interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and tumour-necrosis factor alpha (TNFα) to attack the invading pathogen through the process of inflammation (8, 12). High levels of these combined peripheral cytokines compromise the endothelial cells of the BBB, thus making it leaky and susceptible to higher levels of entering cytokines (8, 14). IL-1β has been shown to play a pivotal role in febrile seizure development through binding interleukin-1 receptors (IL-1R) in microglia, allowing an increased influx into the CNS (8).

The increase of IL-1β in the CNS triggers the enzyme cyclooxygenase-2 (COX-2) which catalyses the conversion of arachidonic acid into prostaglandin E_2 (PGE_2), at the organum vasculosum laminae terminalis (8, 12, 15-18). The organum vasculosum laminae terminalis is surrounded by the preoptic nuclei in the hypothalamus, which lacks a complete BBB, thus allowing PGE_2 to easily enter and stimulate the neural pathways that raise body temperature (8, 15, 19, 20).

The hypothalamus is comprised of 4 regions, the most rostral being the preoptic region, followed by the supraoptic or anterior region, the tuberal region and finally the mammillary region (21). The preoptic nuclei or preoptic area (POA) is found in the rostral part of the hypothalamus and is split into 13 nuclei regions (21). It is most often referred to as the thermoregulatory hub as well as the fever centre as it is this brain area that increases the core body temperature during a fever (22). In order to trigger the fever producing mechanism, PGE_2 acts on prostaglandin EP3 (EP_3R) receptors predominantly in the medial and median preoptic nuclei regions (23). The POA also plays a role in the sleep wake cycle, reproductive and maternal behaviour, as well as cardiovascular responses and fluid homeostasis (21).

In normal non febrile circumstances, there is a negative feedback mechanism provided by the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1Ra) that promotes homeostasis by competitively binding to the same receptors as IL-1β, thus modulating IL-1β mediated production of PGE_2 and subsequently reducing the fever response (8, 15, 22). During febrile seizures however, due to the increased concentration of IL-1β, its binding is favoured over its antagonist IL-1Ra by microglia IL-1R, resulting in its excess influx into the CNS, subsequently ramping up PGE_2 production (8, 10, 14, 24).

Elevated IL-1β concentrations in the CNS and particularly in the brain causes a neurotransmitter imbalance that favours glutamate and inhibits gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter in the hippocampus (8, 16, 25). Glutamate is an excitatory neurotransmitter and exerts its effects by binding to and activating cell surface receptors that open membrane channels allowing charged ions i.e. Ca^{2+}, Cl^-, K^+ and NA^+ to pass through (26). This leads to neuronal hyper-excitability as well as neuronal death due to over activation of the glutamate receptors resulting in excessive neuronal firing rates (8, 16, 25).

THE IMMUNE SYSTEM AND INFLAMMATION

Febrile seizures usually begin with the activation of the immune system resulting from an underlying infection. The human immune system is a complex layered network of organs, tissues, specialized cells and proteins, that protects the body from invading pathogens by eliciting an inflammatory response (27, 28). It is well accepted that the immune system can be divided into two segments: the

innate immune system which is non-specific, and the adaptive immune system, activated by the first, which is specific and also has memory (29-31).

The innate immune system is the first to respond to an invading pathogen e.g. bacterial infections, by employing a group of proteins known as cytokines released from phagocytic immune cells, namely macrophages, neutrophils and dendritic cells (2, 27, 28). These activated cells migrate to the site of injury via chemotaxis, in an innate immune system process known as inflammation (32, 33). Inflammation requires the involvement of these immune cells, surrounding blood vessels, and the released molecular mediator proteins to both eliminate the initial cause of injury as well as the damaged cells, and to initiate tissue repair (33, 34). Immune cells possess surface pattern recognition receptors (PRRs) that allow it to recognise either pathogen-associated molecular patterns (PAMPs) found on various pathogens cell membranes, or damage-associated molecular patterns (DAMPs) found on damaged host cell membranes (33-36). Activation of immune cell PRRs through binding to either PAMPs or DAMPs leads to the recruitment of additional immune cells, and the release of molecular mediator proteins to initiate the classic inflammatory reaction, first recognized by Celsus over 2,000 years ago, i.e. rapid swelling (*tumor*), redness (*rubor*), pain (*dolor*), and heat (*calor*) (35, 37), as well as loss of function (*functio laesa*) later added by Galen. The rapid infiltration of additional immune cells allows for the rapid removal of pathogens, and for tissue repair, thereby returning to a state of homeostasis (29-31). If this first line of response fails, the adaptive immune response is activated (28, 38, 39).

Adaptive immunity, based on antigen-specific responses of T and B lymphocytes, creates a specific immunological memory of the response to that particular pathogen thereby enhancing the body's defence against future insult of that pathogen (28, 38, 39). Depending on the severity of the insult, the adaptive immune system will, or will not be activated during a febrile response, but its involvement will not be considered for the development of a febrile seizure in this study.

Although febrile seizures start with the activation of the immune system, it is the compromised BBB that allows the influx of proinflammatory cytokines into brain tissue that ultimately results in the fever and convulsions experienced during febrile seizures.

THE BBB AND NEUROINFLAMMATION

The neuroimmune system, like the immune system, consists of structures and processes that link the immune system to the central nervous system (CNS), consisting of the brain, spinal cord and the neuroimmune system. The neuroimmune system involves biochemical and electrophysiological interactions to protect neurons from pathogens and disease by maintaining highly selective permeable barriers i.e. the blood brain barrier (BBB), as well as mediating neuroinflammation and wound healing by mobilizing host defences (40, 41). During a febrile seizure, high levels peripheral proinflammatory cytokines compromise the endothelial cells of the BBB, thus making it leaky and susceptible to higher levels of entering cytokines (8, 14).

The key cellular components of the neuroimmune system are glial cells, which include astrocytes, oligodendrocytes, and the most prominent glial cells in the CNS, microglia (42). The concept of Microglia was first introduced in 1932 by Pio del Rio-Hortega in a book titled 'Cytology and Cellular Pathology of the Nervous System' under a chapter titled 'Microglia' which covered his research published in a series of papers between 1919 and 1927 (43, 44). Pio del Rio-Hortega (1932) (45) postulated 9 key aspects of microglia that hold true today, even after much research in the preceding years, i.e. '1) microglia enter the brain during early development. 2) These invading cells have amoeboid morphology and are of mesodermal origin. 3) They use vessels and white matter tracts as guiding structures for migration and enter all brain regions. 4) They transform into a branched, ramified morphological phenotype in the more mature brain (known today as the resting microglia). 5) In the mature brain, they are found almost evenly dispersed throughout the central nervous system and display little variation. 6) Each cell seems to occupy a defined territory. 7) After a pathological event, these cells undergo a transformation. 8) Transformed cells acquire amoeboid morphology similar to the one observed early in development. 9) These cells have the capacity to migrate, proliferate and phagocytose.' Taken from a paper published by Kettenmann et al. (2011) (44). In this way, microglia act as the innate immune cells of the CNS and are the brains first line of defence once the BBB has been compromised i.e. during a febrile infection, and once activated, they migrate and surround the injury site to clear it, much like inflammation throughout the body (34, 46-52). Activated microglial, like leukocytes, release various proinflammatory mediators including arachidonic acid, cytokines, chemokines, complement proteins, and reactive oxygen and nitrogen species that also directly contribute to neuroinflammation and injury (53, 54).

The BBB is a diffusion barrier between the brain and the circulating blood, and is essential for the normal function of the CNS (55, 56). The BBB is made up of specialised endothelial cells with more extensive tight junctions than those found throughout the body, allowing for highly selective transport of molecules, ions, and cells in order to regulate homeostasis, and to provide protection e.g. from toxins, pathogens, inflammation, injury, and even disease (55-58). A compromised BBB, as the result of excess IL-1ß during a febrile seizure, may result in altered signalling, allowing excess immune cell infiltration, leading to neuroinflammation, dysregulation and often degeneration (55-58).

INTERLEUKIN-1 BETA (IL- 1ß)

Eleven ligands make up the interleukin-1 (IL-1) family, including two proinflammatory ligands, interleukin-1 alpha (IL-1α) and IL-1β, and their naturally occurring antagonist, IL-1Ra, that play a central role in the regulation of the immune inflammatory response (59-62). IL-1α and IL-1β both bind to the same receptor molecule IL-1 receptor 1 (IL-1RI), that recruits a necessary coreceptor IL-1 receptor accessory protein (IL-1RAcP) to induce further cascades to initiate inflammation (59, 62, 63).

In order to modulate the inflammatory response, IL-1Ra competitively binds the same IL-1RI, however does not recruit IL-1RAcP, and therefore does not initiate signalling to cause inflammation (24, 59-63). In relation to neuroinflammation experienced during a febrile seizure, excess IL-1β is able to cross the compromised BBB by competitively binding to IL-1RI in the CNS, and trigger COX-2 to induce inflammation and fever through activation of pyretic prostaglandins, particularly PGE_2 (8, 15, 63).

CYCLOOXYGENASE-2 (COX-2) INDUCED PROSTAGLANDIN PRODUCTION

COX-2, an enzyme responsible for the formation of prostaglandins (64) can be found in high concentrations in the CNS, where it participates in the early response to pro-inflammatory mediators and stimuli during an inflammatory response (65-70). Infectious organisms e.g. high levels of proinflammatory cytokines such as IL-1β, stimulate the overexpression of COX-2 in endothelial cells of the BBB (69-73). COX-2 was found by Smith and Lands (1972) (74), and Hamberg et al. (1974) (75), to be the major enzyme involved in the oxidative conversion of arachidonic acid (AA) into prostaglandins. In relation to inflammation and fever, COX-2 catalyses the conversion of arachidonic acid into the pyretic prostaglandin PGE_2, that subsequently induces the preoptic nuclei of the hypothalamus to increase core temperature (15, 16, 19, 67, 73, 76).

Prostaglandins are lipid metabolites produced from the conversion of arachidonic acid by COX-2, and play a major role in inflammation and pain in the body (65, 73, 76). Arachidonic acid, a polyunsaturated fatty acid found in the phospholipid membrane of cells and derived from dietary sources, is converted to prostaglandin H2 (PGH_2) by hydrolysed phospholipids via the enzyme phospholipase A_2, which can be upregulated via hormonal stimulation (65, 73, 77). The exact mechanism by which PGE_2 is synthesised from PGH_2 is not well understood, but it is evident than many prostaglandin synthesised products act in conjunction with cyclooxygenase to produce the target prostaglandins (15, 19, 22, 77-79). The pyrexia-producing PGE_2 is formed by COX-2, as it was also shown by Li et al. (1999) (80) that mice administered with COX-2 inhibitors fail to produce fever when treated with LPS. The pyretic action of PGE_2 is mediated by the EP_3R receptor, as it was shown by Ushikubi et al. (1998) (81) that mutant mice lacking EP_3R do not develop fever after administration of PGE_2.
For the purpose of this study, we measured PGE_2 and its receptor EP_3R concentrations in hypothalamic tissue as a means to identify fever progression, which was taken into consideration when selecting the most appropriate rat model of febrile seizure.

FEVER

"Heat is the immortal substance of life endowed with intelligence. . . . However, heat must also be refrigerated by respiration and kept within bounds if the source or principle of life is to persist; for if refrigeration is not provided, the heat will consume itself"

Hippocrates (82)

Fever, known also as pyrexia or a febrile response, provoked by infection, is thought to provide an optimal hyperthermic environment for enhancing host defences by increasing mobility and enhancing leukocyte phagocytosis, all the while reducing the pathogens viability (21, 76). During fever generation, PGE_2, a powerful endogenous pyrogenic mediator of the POA binds to EP_3R on a population of GABAergic neurons in the POA (22, 83). The binding of PGE_2 to EP_3R is responsible for the febrile response as it was shown by Morrison et al. (2011) that EP3R deletion in neurons distributed in the POA suppressed most of the febrile response to PGE_2 (22). PGE_2 acts to induce fever by inhibiting the firing rate of preoptic warm-sensitive neurons, therefore suppressing heat loss, as well as increasing the firing rate of cold-sensitive neurons to enhance heat production and retention (15). This effectively elevates the hypothalamic set-point temperature, leading to fever (15, 22). Apart from fever, febrile seizures also present with convulsive activity due to excessive neuronal firing driven by the increased influx of IL-1β into the CNS resulting in the imbalance of two key neurotransmitters, i.e. glutamate and GABA.

GLUTAMATE AND GAMMA-AMINOBUTYRIC ACID (GABA) IMBALANCE RESULTS IN CONVULSIONS

Two key neurotransmitters in the brain include glutamate and GABA that control excitatory and inhibitory neurotransmission respectively (84-88). During a febrile seizure, elevated plasma concentrations of IL-1β causes an imbalance between these two neurotransmitters, favouring glutamate, and inhibiting GABA, resulting in neuronal hyperexcitability and death (8, 16, 25, 89, 90). Glutamate is the predominant excitatory neurotransmitter of the adult mammalian brain, and mediates its effects through ionotropic and metabotropic receptor subclasses (84, 91). Ionotropic glutamate receptors are dependent on ionic gradients, and include the fast α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), the slow N-methyl-D-aspartate (NMDAR) receptor, as well as kainate receptors (KAR) (84, 92). Metabotropic glutamate receptors require secondary messenger G-protein coupling in order to translate signals, and often modulate ionotropic receptors (18, 93, 94). In relation to febrile seizures, activation of NMDAR causes an influx of calcium ions (Ca^{2+}) into the neuron after which it is then removed from the cytoplasm by mitochondria (92, 95, 96). However, excess glutamate-stimulated influx of Ca^{2+} and uptake by mitochondria can lead to neuronal damage and eventual cell death, known as excitotoxicity (92, 95, 96).

Under normal circumstances, GABA controls glutamate transmission and acts as a negative feedback mechanism by activating $GABA_A$ receptors to regulate chloride ions (Cl^-), and $GABA_B$ receptors to decreases Ca^{2+} conductance, and increase membrane conductance of potassium ion (K^+) transmission, thereby modulating the effects of glutamate (97-100). GABA, the main inhibitory neurotransmitter in the CNS was first shown by Roberts, Frankel and Udenfriend in 1950 to be derived from the breakdown of glucose to glutamate, which then serves as a precursor to GABA. GABA is often the first

neurotransmitter detected during development, and is thus thought to initially have an excitatory role in early brain development (101, 102), but mostly inhibitory in the mature mammalian brain (86, 88).

Excess IL-1ß produced during febrile seizures however has been shown to decrease levels of $GABA_A$ receptor mediated currents, further establishing an imbalance contributing to seizure generation (18, 97, 100, 103). Elevated plasma IL-1β also alters the hypothalamus–pituitary–adrenal (HPA) axis thus resulting in increased amounts of the glucocorticoid cortisone being released in the plasma, thus exacerbating both fevers and seizures (14). Furthermore, these seizures often exacerbated through prenatal stress, have been shown to lead to cognitive and behavioural deficits later in life, as well as increased susceptibility to epilepsy (8, 14).

PRENATAL STRESS

There is increasing evidence to suggest that the prenatal environment can influence foetal development, immunity, and response to further stressors later in life (10, 104-109). It has previously been shown that high levels of glucocorticoids may influence neurotransmitter release (92), suggesting that stress can increase the risk of developing febrile seizures (110). Exposure to prenatal stress during the third trimester has also been shown to exacerbate febrile seizures in rat offspring (14).

Stress, as described by McEwan generally refers to experiences that cause feelings of anxiety and frustration (111). Prenatal stress accounts for all types of stress, i.e. emotional and or physical stress experienced by pregnant mothers, with the most prominent effects occurring during the last trimester (10, 14, 112). Prenatal stress has also been shown to lead to generalized anxiety, depression, deficits in attention and learning, and more serious conditions such as autism and schizophrenia (107, 113-116). Prenatal stress is also associated with an increased risk of premature birth (117), and emotional and cognitive deficits in early life (10, 118-120). Women exposed to stress during pregnancy are also themselves at higher risk of developing various diseases and psychological distress (107, 108, 120, 121). The primary hormones responsible for regulating the stress response in humans are glucocorticoids and adrenaline, produced by the HPA axis, stimulating the body's "fight or flight" response (122-124).

THE HPA-AXIS AND THE SYNTHESIS OF CORTISOL

The HPA axis, a major neuroendocrine system consists of three components, the hypothalamus, pituitary gland, and the adrenal glands that work together via direct influences and feedback interactions (125, 126). The HPA axis plays a central role in regulating homeostatic systems in the body, i.e. the metabolic, cardiovascular, immune, reproductive and central nervous systems (125, 126). In maintaining these systems, it thereby regulates bodily processes such as digestion, mood and emotions, sexuality, and most importantly it controls the body's reaction to stress allowing for adaptation and ultimately survival (125, 126).

Upon a stressor e.g. prenatal stress, the paraventricular nucleus within the mothers hypothalamus releases the neurohormone corticotrophin-releasing hormone (CRH), into the hypophysial portal blood vessels connecting the hypothalamus and the pituitary gland (127, 128). This neurohormone then stimulates the anterior pituitary gland to produce and secrete adrenocorticotropic hormone (ACTH) into the blood circulation (127, 128). The ACTH activates glucocorticoid synthesis and release from the cortex of the adrenal glands, which are found atop the kidneys (128). Glucocorticoids bind to glucocorticoid and mineralocorticoid receptors found throughout the CNS, which under normal circumstances acts as a negative feedback mechanism to control glucocorticoid concentrations by inhibiting further cortisol release (127, 128).

Circulating glucocorticoids are predominantly protein bound to corticosteroid binding globulin, but at high stress levels, binding proteins become saturated, resulting in high levels of free glucocorticoid that continuously binds and activates its receptors (127). The altered, over stimulated HPA axis then often malfunctions, leading to excess glucocorticoid production (107, 109). Glucocorticoids are lipophilic, and can therefore freely cross the placenta, especially once it has become leaky due to decreased 11beta-hydroxysteroid-dehydrogenase 2 (11β-HSD-2) concentrations (14, 118).

ROLE OF PLACENTAL 11ß -HSD-2

Under normal circumstances, foetal glucocorticoid levels are about 10-fold lower than maternal levels, due to the actions of placental 11β-HSD-2, an enzyme that converts cortisol (corticosterone in rats) to its inactive form cortisone (dehydrocorticosterone in rats) (127, 129). The concentration of 11β-HSD-2 increases with advancing gestation thus limiting foetal glucocorticoid exposure during critical stages of development (127). The concentration of 11β-HSD-2 however decreases during the third trimester in preparation for parturition and lung maturation, thus allowing an increased influx of the mother's glucocorticoids into the foetus (14, 118, 127). Excess cortisol exposure in utero is associated with increased activity of the offspring's HPA axis (127), thus higher maternal cortisol levels in the third trimester are associated with increased cortisol response in the new-born to light stress (118, 127).

Maternal malnutrition, stress or illness is further associated with down regulation of 11β-HSD-2 mRNA expression thus increased placental glucocorticoid sensitivity, further allowing cortisol entry to the foetus (127). Due to increased levels of the cortisol perforating though the placental barrier, the developing foetus's HPA axis may become dysregulated (105, 107, 108). This dysfunction of the HPA axis leaves the new-born more sensitive to future stress, resulting in even higher cortisol exposure or greater cortisol burden following each stressful episode (118, 121, 127, 128) leaving the neonate offspring more prone to infection than non-prenatally stressed neonates (76, 107).

As exposure to prenatal stress during the third trimester has been shown to exacerbate febrile seizures in rat offspring, we also included a stress factor in this study to further test this theory (14). Prenatal stress can be mimicked in rat models by exposing pregnant dams in their third trimester to various forms of stressors (112). Examples of stressors for pregnant rats range from food deprivation, foot-shock, rodent restrainers to more invasive methods such as exogenous glucocorticoid injection (112). Restraint is the most popular form of stressor used in research as it can be easily adapted, and is one of the few methods that also directly stress the foetus by limiting its movement (115, 130).

ANIMAL MODELS AND WHY WE USE THEM

In order to fully investigate the potential consequences of febrile seizures, the human condition is mimicked using animal models, the most commonly used being Sprague-Dawley or Wistar rats (10, 14, 118, 131, 132). According to the Foundation for Biomedical Research (FBR, 2018)(133), mice and rats are used for 95% of all laboratory animal research (133). This is due to their similar human resemblance, and their convenient housing due to their small size. Rodents reproduce quickly and have a short lifespan allowing several generations to be observed in a relatively short period of time. According to the National Human Genome Research Institute (NHGRI, 2018, genome.gov), mice and rats that are used in medical trials are usually inbred, allowing them to be almost genetically identical, making the results uniform. Rodents genetic, biological and behavioural characteristics closely resemble those of humans, thus allowing many human conditions to be replicated for study.

ANIMAL MODELS OF FEBRILE SEIZURE

Various methods have been employed to mimic the conditions of febrile seizures, ranging from administration of numerous drugs, electrical stimulation, and heated environments (10, 14, 118, 131, 134, 135). In order to correctly mimic febrile seizures, two disease states need to be simulated, i.e. a fever, followed by seizure activity, expressed as convulsions. Lipopolysaccharide (LPS), the major component of the outer wall membrane of Gram-negative bacteria functions to increase the structural integrity of the bacteria and to protect the membrane by increasing the negative charge of the cell therefore stabilizing the overall membrane structure (136).

LPS induces a strong defensive response from animal immune systems, thus injecting small amounts can mimic a systemic infection, leading to a fever-like situation (10, 14, 131, 136, 137). LPS is easily obtainable, inexpensive, easy to work with, and systemic injection triggers the release of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α), IL-6 and IL-1β and is thus the most commonly used method to mimic infection and fever in rodent models (8, 12, 136). LPS induces fever by triggering COX-2 which catalyses the conversion of arachidonic acid to PGE2, thereby causing the increase in the set point temperature (8, 67). It has been shown that systemic injection of LPS primes the brain to respond more rapidly to further insult thus producing more severe seizures (137).

For this study we used varying doses of LPS in conjunction with a chemoconvulsant to induce febrile seizure in young rats. Our model was based on the Heida et al. (2005) model whereby febrile seizures were induced in 14-day old rats with a combination of LPS and kainic acid (KA) (138). KA is an excitotoxic, convulsive neurochemical and potent neurotoxin derived from the algae Digenea simplex that causes immobility, an increased incidence of wet dog shakes, as well as long-lasting generalized tonic-clonic convulsions (139). KA produces tissue damage and lesions that are consistent with those observed in human patients with temporal lobe epilepsy (140). KA is thus used to mimic seizures experienced by children suffering from febrile seizures (10, 14, 118, 131). Intraperitoneal (i.p.) injection of KA, a natural analogue to glutamate (141) has been shown to induce febrile seizures by inducing a glutamate/GABA imbalance by comprise the ionotropic glutamate receptors (142, 143). KA binds to activated kainic acid receptors (KAR), which have been shown to have a high affinity for glutamate (141), acting as a specific high affinity agonist (140, 141, 144), therefore increasing glutamate binding and evoking seizures (141, 142, 145). Structural changes to glutamate receptors can affect their calcium permeability, which can lead to accelerated calcium entry into cells, thereby accelerating mitochondrial damage and ultimately cell death (146, 147).

Heida et al. (2005) obtained febrile seizures in at least 50% of animals without recurrent seizures using 200μg/kg of LPS followed 2 h 30 min later by 1.75mg/kg of KA administered intraperitoneally (2). This model proved clinically significant with regards to fever, and seizure duration of at least 60 minutes and is therefore considered a favourable model in many research laboratories (10). Previous work done in our laboratory using this model however induced febrile seizures in 100% of animals treated, with many of our animals experienced reoccurring severe tonic-clonic convulsions with a high mortality rate, making its use daunting to students.

Extended exposure to a hyperthermic environment (H) can adversely affect brain health, body performance and comfort, and in extreme cases can lead to death (148). Prolonged febrile seizures have been be initiated by maintaining a core temperature of 38.5-42.5 ^{o}C for approximately 30 minutes (149-151). Core temperatures of 38.5 to 42.5 ^{o}C corresponds to threshold temperatures that would be required to evoke complex febrile seizures in human neonates (149-151). Holtzman et al. (1981) (152) made use of a heated chamber, Hjeresen et al. (1983) (153) made use of a microwave, Dubé et al. (2000) (154), Koyama et al. (2012) (132) and Tao et al. (2016) (155) made use of a hair dryer, whereas Jiang et al. (1999) (156) and Yagoubi et al. (2015) (149) made use of a hot water bath.

Briefly, in each case young rats ranging from 10 to 15 days old were placed in heated environments for a brief period to increase core body temperature. In each case, as soon as signs of seizure occurred, the rats were removed from the hyperthermic environment and observed for seizure activity (132, 149, 152, 154). The pups were then returned to their dams once seizures ceased and body temperatures returned to normal (132, 149, 152, 154). Seizures induced in rats by H are usually more subtle than those induced by

chemoconvulsants, but are identifiable through behavioural symptoms such as sudden arrest of hyperthermia-induced hyperactivity, facial automatism, forelimb clonus and tonic body flexion (157, 158).

AIMS AND OBJECTIVES

For this study we mimicked febrile seizures on postnatal day (PND) 14 Sprague Dawley pups using models established by Heida et al. (2005) (2), and a novel model to our laboratory by Yagoubi et al. (2015) (149). The Heida et al. (2005) model of LPS and KA was selected for its accuracy in mimicking both the fever and the convulsions in a similar manner experienced by young children, however it yielded a high mortality rate. The aim of this study was thus to refine the current model by lowering the dose of both LPS and KA in an attempt to lower the high mortality rate experienced with this model, all while maintaining successful seizure outcomes. A second goal of this study was to introduce and optimise an alternate less invasive febrile seizure model, a hyperthermic model by Yagoubi et al. (2015) (149) for use in our laboratory.

The focus of this study was thus to investigate:

1. Determine the most effective and efficient dose of LPS and KA to induce febrile seizures in rats, while maintaining a high survival rate.
2. Determine the most effective and efficient LPS-KA and hyperthermia-based animal models of febrile seizure to be used in our laboratory.
3. Assess the neurochemical effect of prenatal stress on PGE_2 and its receptor EP_3R concentration in hypothalamic tissue of LPS-KA and hyperthermia based animal models of febrile seizure.

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CHAPTER TWO

ARTICLE

THE EFFECTS OF PRENATAL STRESS ON HYPOTHALAMIC PGE_2 AND ITS RECEPTOR CONCENTRATIONS IN RAT MODELS OF FEBRILE SEIZURE

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RESEARCH PAPER

The Effects of Prenatal Stress on Hypothalamic PGE_2 and its Receptor Concentrations in Rat Models of Febrile Seizure

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Key words: febrile seizure; prenatal stress; hypothalamus; prostaglandin E_2; prostaglandin EP_3 receptor

Abbreviations: cyclooxygenase-2 (COX-2), prostaglandin EP3 receptors (EP_3R), gamma-aminobutyric acid (GABA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), gestational day (GND), hypothalamic-pituitary-adrenal (HPA), kainic acid (KA), lipopolysaccharide (LPS), non-stressed (NS), prostaglandin E2 (PGE_2), stress (S)

HIGHLIGHTS

- A lowered dose of LPS (50 μg/kg) and KA (0.44 mg/kg), was capable of inducing seizures while maintaining a high survival rate.
- All rat pups exposed to a heated environment for a period of time experienced mild to moderate seizures, ranging from facial twitching to jerking and loss of posture.
- Prenatal stress lead to a decreased expression of PGE_2 in the hypothalamus of febrile seizure animals.
- Prenatal stress increased seizure severity, and hypothalamic EP_3R concentrations.

ABSTRACT

Background: Febrile seizures are the most common type of seizure experienced by 3 months to 6-year-old children. Proinflammatory cytokines, often triggered by simple infections, are known mediators of fever, and have been implicated in febrile seizure onset. Of particular interest is the mechanism involving fever through prostaglandin E_2 (PGE_2) production, and prenatal stress which is prominent in third world countries, and has been shown to exacerbate febrile seizures in offspring. Animal models mimicking febrile seizures can result in 50% seizure onset or yield high mortality rates, therefore the aim of this study was to refine two existing models of febrile seizure to yield greater % of seizure onset with low mortality.

New method: Pregnant Sprague Dawley dams were exposed to prenatal stress, and febrile seizures induced in their subsequent offspring on postnatal day 14 by one of two refined models. We refined the Heida et al. (2005) 200 µg/kg lipopolysaccharide (LPS) and 1.75 mg/kg kainic acid (KA) model and adapted a novel hyperthermic (H) model for use in our laboratory.

Results: Dosage was reduced to 50 µg/kg LPS and 0.44 mg/kg KA which significantly decreased mortality, while yielding 100% convulsions. The adapted H model produced 100% successful convulsions. Additionally, exposure to prenatal restraint stress significantly exacerbated seizure severity.

Comparison with Existing Method(s): The modified LPS-KA and H models successfully induced convulsions with drastically reduced mortality rates compared to existing models.

Conclusion: The modified LPS-KA model was selected as the most effective and most efficient model.

1 INTRODUCTION

A febrile seizure is a neurological abnormality that presents with neuronal hyper-excitability and convulsions, usually occurring after a fever due to an underlying systemic infection i.e. gastrointestinal and upper respiratory tract infections, or middle ear infections (1-3). Triggered by these infections, febrile seizures are thought to be linked to the activation of the immune system through the release of pro-inflammatory cytokines, predominantly interleukin-1 beta (IL-1β), interleukin 6 and tumour-necrosis factor alpha (4-7). The increased IL-1β influx triggers the enzyme cyclooxygenase-2, which catalyses the conversion of arachidonic acid into prostaglandin E_2 (PGE_2), that subsequently triggers the preoptic nuclei region of the hypothalamus to increases core body temperature (8-15). In order to induce fever, PGE_2 acts on prostaglandin EP_3 receptors (EP_3R) predominantly in the medial and median preoptic nuclei regions of the hypothalamus (16-18). The exact mechanism by which PGE_2 is synthesised is not well understood, but it is evident than many prostaglandin products act in conjunction with cyclooxygenases to produce target prostaglandins (2, 11, 12, 19, 20).

Various animal models have been developed to mimic the conditions of febrile seizures, ranging from the administration of drugs, electrical stimulation, or heated environments (3, 21-25). One of the drug induced febrile seizures models is the Heida et al. (2005) (26) injection of gram negative bacterial endotoxin lipopolysaccharide (LPS) (200 μg/kg) followed 2 h 30 min later by a sublethal dose of kainic acid (KA) (1.75 mg/kg) (26). Holtzman et al. (1981) (27) made use of a heated chamber, Hjeresen et al. (1983) (28) made use of a microwave, Dubé et al. (2000) (29), Tao et al. (2016) (30) and Koyama et al. (2017) (31) made use of a hair dryer, whereas Jiang et al. (1999) (32) and Yagoubi et al. (2015) (33) made use of a hot water bath. Briefly in each case, young rats ranging from 10 to 15 days old were placed in heated environments for a brief period to increase core body temperature. In all cases, as soon as signs of seizure occurred, the rat pups were removed from the heated environment, and observed for seizure activity (27, 29, 31, 33). Furthermore, exposure to prenatal stress during the last trimester has been shown in our laboratory by Qulu et al. (2012) (3) to exacerbate febrile seizures in rat offspring through dysregulation of the offspring's hypothalamic-pituitary-adrenal axis (3, 23, 34).

Previous work done in our laboratory by Qulu et al. (2012; 2015) (3, 22), Cassim et al. (2015) (23), and Mkhize et al. (2017) (35) using the Heida et al. (2005) (26) model induced febrile seizures in 100% of animals treated, in contrast to at least 50% experienced by the Heida et al. (2005), and had a high mortality rate, especially when coupled with prenatal stress (3, 22, 23, 35, 36). Prenatal stress has been shown to result in the dysregulation of the foetal HPA axis, thus allowing increased entry and exposure of the foetus to maternal cortisol (37). Cortisol in excess results in an increased proinflammatory response, leaving the new-born more prone to infection than non-prenatally stressed neonates (38-43). Many of our animals experienced reoccurring severe tonic-clonic convulsions with a

high mortality rate. The model itself however has proven to be of vital importance in studying febrile seizures due the accurate portrayal of the disorder, i.e. fever due to proinflammatory cytokine release, followed by neuronal hyperexcitability (3, 22, 23, 26, 35, 36, 44, 45). However, to effectively reduce the mortality rate associated with this model, the aim of our study was to investigate the most effective and efficient dose of LPS and KA to induce febrile seizures, while maintaining a high survival rate. We also aimed to introduce and refine a new model based on work done by Jiang et al. (1999) (32) and Yagoubi et al. (2015) (33), and thus set out to determine the most effective and efficient febrile seizure rat model to use in our laboratory. As with our previous studies, febrile seizures were induced in 14-day old Sprague Dawley rat pups.

2 MATERIALS AND METHODS

2.1 MATERIALS

Lipopolysaccharide (LPS) (Escherichia coli O111:B4 phenol extraction, lyophilized powder) and kainic acid (KA) (kainic acid monohydrate K0250, dry powder) were obtained from Sigma-Aldrich, (Gauteng, South Africa). Both drugs were dissolved in saline solution (0.9 %; 10 ml/kg) and injected intraperitoneally (i.p.) using a 29 G 0.5 mm insulin needle. Elabscience ELISA kits were obtained from Anatech, Gauteng, South Africa. The Zymo Research Quick-RNA™ Miniprep Kit RNA extraction kit was obtained from Inqaba Biotechnical Industries, Pretoria, South Africa. The iScript™ cDNA synthesis kit and the iTaq™ universal SYBR Green Supermix, both from Bio-Rad were obtained from Lasec, Cape Town, South Africa.

2.2 BREEDING ANIMALS

All procedures were performed under ethical clearance (AREC/045/018M) from the University of KwaZulu-Natal Research Committee, Animal Ethics sub-committee. A total of 15 female and 5 male Sprague Dawley (SD) rats were obtained from the Biomedical Resource Unit of the University of KwaZulu-Natal, (6 females for the preliminary toxicity study, and 8 females for the rat model comparison study), a total of 72 pups were used. Rats were group housed under standard laboratory conditions of 22±1°C room temperature, 70 % humidity, and a 12 h light/dark cycle (lights on at 06:00 h, off at 18:00 h). Food and water were available *ad libitum*.

2.3 MATING AND PRENATAL STRESS PROTOCOL

Female SD rats were paired and allowed a week to acclimatise to minimise stress, and synchronise oestrous cycles. Thereafter, vaginal smears were done daily to assess the female's oestrus cycle, and a male rat introduced during proestrus for mating (46). Vaginal smears were performed to determine successful mating through the presence of sperm in the smear, after which the male rat was removed (46). On gestational day (GND) 14, pregnant females were divided into 2 groups, non-stressed (NS) left undisturbed in their home cages, and stressed (S). To induce stress, female dams were taken daily to a

separate room and placed in rodent restrainers for 1 h a day, for a total of 7 days (3, 22). All stress procedures were carried out between 11:00 h and 12:00 h. The S pregnant rats were returned to their home cages at the end of the stress period each day.

2.4 STUDY A: PRELIMINARY DOSE OPTIMIZATION

In this study we compared different dose of LPS and KA to effectively induce febrile seizures, while maintaining a high survival rate.

2.4.1 POSTNATAL HANDLING

On postnatal day (PND) 14, at 10:00 h, the NS and S pups were removed from their dams and separated into 5 groups as shown in Table 2.1 (n = 3/group). To induce febrile seizures the Heida et al. (2005) (26) model was modified; each group was injected i.p. with a step-wise reduced percentage of the total dose of LPS (200 µg/kg) followed 2 h 30 min later by the same step-wise reduced percentage of the total dose of KA (1.75 mg/kg) to induce febrile seizures. LPS and KA were both dissolved in saline solution (0.9 %; 10 ml/kg) and injected i.p. The controls were injected with saline twice in the same manner as the experimental groups to account for injection stress.

Table 2.1 Study A. Reduced LPS and KA doses. n=3/group.

	Non-Stressed		Stressed	
1. Control	Saline 10 ml/kg	Saline 10 ml/kg	Saline 10 ml/kg	Saline 10 ml/kg
2. 25%	LPS 50 µg/kg	KA 0.44 mg/kg	LPS 50 µg/kg	KA 0.44 mg/kg
3. 50%	LPS 100 µg/kg	KA 0.88 mg/kg	LPS 100 µg/kg	KA 0.88 mg/kg
4. 75%	LPS 150 µg/kg	KA 1.31 mg/kg	LPS 150 µg/kg	KA 1.31 mg/kg
5. 100%	LPS 200 µg/kg	KA 1.75 mg/kg	LPS 200 µg/kg	KA 1.75 mg/kg

2.4.2 BEHAVIOURAL ANALYSIS

2.4.2.1 ASSESSMENT OF TEMPERATURE

Oral temperature (ºC) was measured with a Medic Thermometer Digital Flexi Tip (110442000EA, Dis-Chem, South Africa) at least 15 min after removing pups from their dams in order to minimise an increased reading due to feeding. Recordings were taken for each pup prior to the first injection, and then again 15 min after the second injection.

2.4.2.2 ASSESSMENT OF FEBRILE SEIZURE SEVERITY

Febrile seizures were video recorded and assessed for 1h 30 min after the injection of KA, the footage was loaded onto the behavioural analysis programme BORIS (47) (version 6.3.5) and the behaviour

rated by third party candidates blinded to the treatments. The behaviour was scored a severity rate of 0 – no response, to 5 – repeated severe tonic-clonic convulsions on the Racine scale shown in Table 2.2.

Table 2.2 Seizure severity was assessed using the following Racine scale (3, 5, 48).

Stage	Behavioural Response
0	No response
1	Ear and facial twitching
2	Loss of postural control
3	Myoclonic jerks and rearing
4	Clonic convulsions – animal falling on its side
5	Repeated severe tonic-clonic convulsions

2.4.3 TISSUE COLLECTION AND ANALYSIS

On PND 16, at 10:00 h, a sharp guillotine was used to decapitate the rat pups. Hypothalamic tissue was collected in 1.5 ml nuclease-free Eppendorf tubes, snap frozen in liquid nitrogen and then stored in a bio freezer at -80 °C for further analysis.

2.4.3.1 PROSTAGLANDIN E_2 (PGE_2) CONCENTRATION ANALYSIS

PGE_2 concentrations were assessed in hypothalamic tissue by means of competitive enzyme-linked immunosorbent assay (ELISA). The ELISA was performed using the Elabscience PGE_2 (Prostaglandin E_2) ELISA Kit (E-EL-0034) (Elabscience, Wuhan, China) according to the manufactures guidelines. In short, frozen samples were thawed on ice, diluted (1:9) in ice-cold phosphate-buffered saline (PBS) (pH 7.4) and homogenised on ice using a sonicator (Qsonica, MODEL CML-4), centrifuged (Hermle LASEC, Germany) at 5000 x g and the supernatant collected for the assay. The assay procedure was performed in duplicate for each standard and each sample following the manufactures guidelines. The plate was washed using a BioTek ELx50 plate washer (BioTek, Highland Park, USA), incubated at 37°C, and optical density (OD) read at 450 nm in a SPECTROstar Nano (BMG, Labtech Ortenberg, Germany) absorbance plate reader.

2.4.3.1.1 Calculations

The average (avg.) OD was calculated for each standard and sample, then correct by subtracting the avg. blank (B) OD value as in Equation 2.4.3.1.

Equation 2.4.3.1 $$corrected\ OD\ value = avg.OD - avg.B\ OD$$

The corrected standard OD values were inserted into Graph Pad Prism version 7 (GraphPad Software Inc., California, USA) to obtain a linear regression graph. The X values of the linear regression graph

were used to interpolate sample concentrations. The concentrations were subjected to column statistics to determine the distribution, and thereafter further analysed to determine statistical significance.

2.5 STUDY B: EFFECTS OF PRENATAL STRESS ON RAT MODELS OF FEBRILE SEIZURE

In this study we compared the effectiveness of two febrile seizure models. A preselected dose established from the preliminary dose study was used for the drug induced model, and a hyperthermic model adapted for use in our laboratory.

2.5.1 PRENATAL HANDLING

Pregnant Sprague Dawley rats were separated into non-stressed (NS) and stressed (S) groups, prenatal stress was induced on GND 14 using the method described in study A section 0.

2.5.2 POSTNATAL HANDLING

On PND 14, at 10:00 h, the NS and S pups were removed from their dams and separated into 3 groups (n = 7/group). Pups received either a combination LPS and KA, or they were exposed to a heated environment to induce febrile seizures. Control animals were injected i.p. with saline, in the same manner as the LPS-KA animals to account for injection stress.

2.5.2.1 LPS-KA MODEL

The preselected dose of LPS and KA from study A was used to induce febrile seizures in study B, i.e. 50 µg/kg of LPS dissolved in saline solution (0.9%; 10 ml/kg) injected i.p., followed 2h 30 min later by a second i.p. injection of 0.44 mg/kg KA, dissolved in saline solution (0.9%; 10 ml/kg). The change in oral temperature and seizure severity was assessed in the same manner as described in Study A, section 2.4.2 Seizure progression was scored using the Racine scale in Table 2.2 above.

2.5.2.2 HYPERTHERMIC MODEL

The hyperthermic environment (H) model, based on previous models by Jiang et al. (1999) (32) and Yagoubi et al. (2015) (33), was refined to make it suitable for use in our laboratory. A well ventilated clear hard plastic (polypropylene) 350 ml container was used to house the rat pup, in place of a glass container. Ventilation was achieved through small holes drilled into the container lid, as well as the upper rim of the container, as a ventilated lid alone proved to be insufficient. The bottom of the plastic container was lined with 2 cm thick polystyrene, which conducted little heat, thus prevented the pup's paws from burning. The container with the pup (n = 1/container) securely enclosed was submerged into a water bath maintained at 60 °C ± 1 °C for about 20 min to allow for a rise in their core temperature. Care was taken to ensure no water entered the container. Once a myoclonic jerk, was experienced, the pup was removed from the container and oral temperature measured before transferring the pup back to room temperature, where it was assessed and scored for further convulsions. Pups were cooled with cold water, rehydrated, dried off, then returned to their home

cage. Containers were well cleaned with 70% ethanol after each test. Pups removed from the heated environment before stage 3 myoclonic jerks failed to display further seizure activity.

2.5.3 TISSUE COLLECTION AND ANALYSIS

A sharp guillotine was used to decapitate the rat pups on PND 16, at 10:00 h. Hypothalamic tissue was collected in 1.5 ml nuclease-free Eppendorf tubes, snap frozen in liquid nitrogen and stored in a bio freezer at -80 °C for further analysis.

2.5.3.1 PROSTAGLANDIN E_2 (PGE_2) CONCENTRATION ANALYSIS

PGE_2 concentrations were assessed in hypothalamic tissue by ELISA in the same manner as described in Study A, section 2.4.3.1. The ELISA was performed using the Elabscience PGE_2 (Prostaglandin E2) ELISA Kit (E-EL-0034) (Elabscience, Wuhan, China) according to the manufactures guidelines. The same calculations described in Study A, section 2.4.3.1.1 were used in order to calculate the sample PGE_2 concentrations.

2.5.3.2 PROSTAGLANDIN E_2 RECEPTOR (EP_3R) CONCENTRATION

Prostaglandin EP_3 receptor (EP_3R) expression was quantified in hypothalamic tissue by means of Real-Time Polymerase chain reaction (qPCR). Primers for the target gene EP_3R and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed on Primer-BLAST (sequence shown in Table 2.3), and were obtained from Inqaba Biotechnical Industries (Pty) Ltd. Primers were diluted to a 100mM stock solution by adding nuclease-free water as per manufacturer instructions.

Table 2.2 Study B. PCR Target and Reference primers. Primers were selected to be 20 base pairs long, falling within the 18-22 base MIQE suggested length. Primers were chosen to have similar melting temperatures between 55-60 ºC, and minimal primer dimer formation opportunities (49).

Primer	Forward	Reverse	NCIB number
EP_3R	ATACCTGCTTCCCTGAGTAT	GAGGCCGAAAGAAGATACAA	NM_012704.1
GAPDH	AGTGCCAGCCTCGTCTCATA	GATGGTGATGGGTTTCCCGT	NM_017008.4

The qPCR analysis was carried out in accordance to the corresponding manufactures guidelines for the 3 major steps, i.e. 1.) RNA isolation using the Zymo Research Quick-RNA™ Miniprep Kit (ZAR1054), 2.) cDNA synthesis using the Bio-Rad iScript™ cDNA Synthesis Kit (1708891), and 3.) amplification using the Roche iTaq™ Universal SYBR Green Supermix (1725120). To isolate RNA, samples were thawed and homogenized (Qsonica, MODEL CML-4) on ice, and then purified with an in-column DNase I treatment to remove all trace DNA. Eluted RNA concentrations were measured for purity using a Nanodrop 2000 (Thermo Scientific, Roche, South Africa). A purity ratio (A260/A280) of 1.7 – 2.1 was considered sufficient for conversion to cDNA. RNA was diluted with nuclease-free water to 50 ng/µl and converted to cDNA in a thermocycler 2.0 (Roche, South Africa) on the same

day in white 8-strip clear capped PCR tubes (Whitehead Scientific, Cape Town, South Africa) then stored at -20 °C to be used for amplification the following day.

For amplification , the target and reference primer stock solutions were diluted 1:20 to form the working dilution (50 ng/ul) for amplification. The reaction mix was prepared in accordance with the Roche SYBR Green Supermix and run in duplicate in a Roche LightCycler96 (Roche, South Africa). The reaction mixture and amplification procedures were carried out in white 8-strip clear capped PCR tubes (Whitehead Scientific, Cape Town, South Africa).

2.5.3.2.1 Calculations

Gene fold expression of EP_3R relative to the reference GAPDH was calculated using the $2^{-(\Delta\Delta Cq)}$ method (49). The avg. quantification cycle (Cq) values were calculated for each sample containing the target gene (EP_3R), and for the same samples containing the reference gene (GAPDH). The change in Cq for each sample including the controls were then calculated by subtracting the reference avg. Cq from the target avg. Cq, as shown in Equation 2.5.3.2. The avg. double change in Cq value was then calculated by subtracting the Cq change of the control samples from the Cq change of the experimental samples as shown in Equation 2.5.3.2. The double change values calculated in Equation 2.5.3.2 were then put to the negative power of 2 in order to calculate the fold change $2^{-(\Delta\Delta Cq)}$ values used for further statistical analysis, as shown in Equation 2.5.3.2 (49).

Equation 2.5.3.2 $$avg.\Delta Cq = target\ avg.Cq - refrence\ avg.Cq$$

Equation 2.5.3.2 $$avg.\Delta\Delta Cq = sample\ avg.\Delta Cq - control\ avg.\Delta Cq$$

Equation 2.5.3.2 $$2^{-(\Delta\Delta Cq)} = 2^{-(\text{Equation 2.5.3.2})}$$

The $2^{-(\Delta\Delta Cq)}$ values were inserted into Graph Pad Prism version 7 (GraphPad Software Inc., California, USA). The data were subjected to column statistics to determine distribution, and thereafter further analysed to determine statistical significance. For the target EP_3R standard curve: y = -3.4488; y-int = 32.66; R_2 = 0.99; efficiency = 1.95. For the reference GAPDH standard curve: y = -3.5338; y-int = 27.08; R_2 = 0.88; efficiency =1.92.

2.6 DATA ANALYSIS

All datasets were analysed using the statistical software programme Graph Pad Prism version 7 (GraphPad Software Inc., California, USA). Gaussian distributions were determined using the Shapiro-Wilk normality test on all data sets before further analyses. Where Gaussian distributions were evident, parametric two-way analysis of variance (ANOVA) tests were performed, followed by Tukey-Kramer post hoc tests. Seizure severity and survival rate data were analysed by Kruskal-Wallis tests followed by Dunn's multiple comparisons test. Correlation tests were performed where stated, and significant p values, and Pearson correlation r values reported. An n = 3 animals were assessed per group for Study A, and an n = 7 animals were assessed per group for study B. $P < 0.05$ was considered statistically significant. Data are presented as mean values, with standard error of the mean (SEM).

3 RESULTS

3.1 STUDY A: PRELIMINARY DOSE OPTIMIZATION

To assess an effective dose of lipopolysaccharide (LPS) and kainic acid (KA) for febrile seizure induction, a dose assessment test was conducted for non-stressed (NS) and stressed (S) PND 14 rat pups receiving varying doses of LPS and KA, e.g. 25% - 100% of the original dose (LPS 200 µg/kg, KA 1.75 mg/kg). The following 5 groups were assessed: (NS-C; S-C), (NS-25%; S-25%), (NS-50%; S-50%), (NS-75%; S-75%), and (NS-100%; S-100%). The average change in temperature, seizure severity and survival for each group was analysed for statistical significance.

3.1.1 TEMPERATURE AND SEIZURE SEVERITY OF FEBRILE SEIZURE DOSAGE GROUPS

An overall dose-dependent decrease in oral temperature ($F_{(4, 20)} = 19.84$; $p < 0.0001$), as well as an overall dose-dependent increase of seizure severity ($F_{(4, 20)} = 71.83$; $p < 0.0001$) was observed. Overall, a greater drop in oral temperature significantly correlated with an increase in seizure severity for both the NS pups (NS temperature vs NS seizure severity p = -0.0074; r = -0.966) and the S pups (S temperature vs S seizure severity p= -0.0259; r = -0.922). A greater overall change in temperature occurred for the 100% dose when compared to the 25% dose, who experienced smaller changes in temperature.

A dose effect was observed as the NS-25% pups experienced facial twitching with loss of postural control which was significantly less severe than the NS-100% pups that experienced clonic convulsions with many pups falling on their side (NS-25% vs NS-100% p = 0.0014).

A significant overall stress effect on seizure severity was observed ($F_{(1, 20)} = 5.444$; $p = 0.0302$), as all S pups experienced more severe seizures when compared to their NS counterparts, i.e. on average the S-25% pups experienced myoclonic jerks coupled with loss of postural control, whereas the S-100% pups all reached stage 5 repeated severe tonic-clonic convulsions (S-25% vs S-100% p = 0.0342). As dosage was increased, temperatures dropped significantly, which correlated with more severe seizures, which often resulted in the death of the animal. Data presented as mean ± SEM in Figure 3.1

			Stressed	
	Change in oral Temperature	Average Seizure Severity	Change in oral Temperature	Average Seizure Severity
Control	-0.73°C	0.00	-0.60°C	0.00
25%	-2.97°C	2.33	-2.50°C	3.33
50%	-3.43°C	3.33	-3.10°C	3.67
75%	-3.27°C	4.00	-2.63°C	4.67
100%	-4.47°C	4.67	-4.17°C	5.00

Study A: Preliminary Toxicity Test Behavioural Results. Change in Oral Temperature, Seizure Severity and Survival. Initial and post treatment oral temperature was measured for each pup, thereafter seizers were scored 0 – no response, to 5 – repeated severe tonic-clonic convulsions on a Racine scale. Survival was regarded as surviving until sacrifice. n = 3/group.

Preliminary Dose Test: Correlation between Oral Temperature and Seizure Severity.

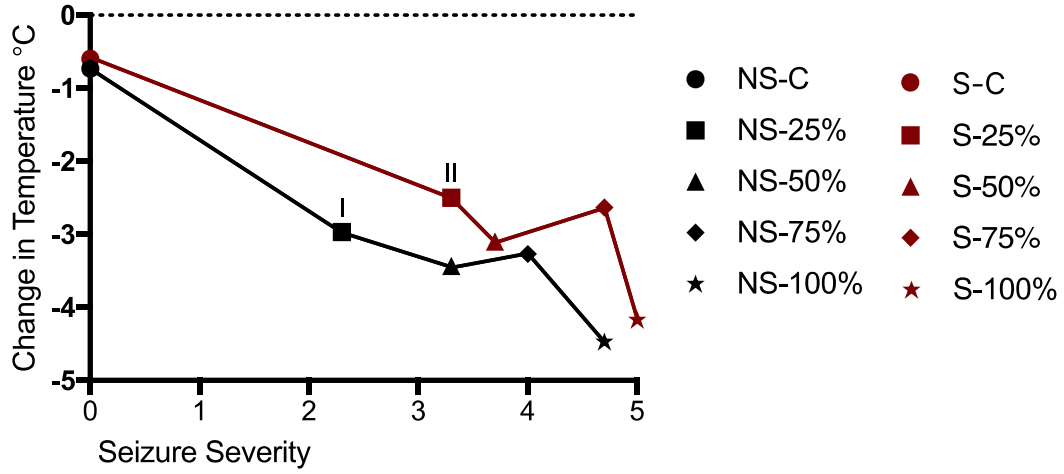

Figure 3.1 Study A: Graph depicting change in oral temperature and seizure severity of NS and S animals receiving different doses of LPS-KA. A high correlation was evident for NS (NS temperature vs NS seizure severity $p = 0.0074$; $r = -0.966$), and S pups (S temperature vs S seizure severity $p = 0.0259$; $r = -0.922$). I $p < 0.05$ compared to NS-100%. II $p < 0.05$ compared to S-100%. Data presented as mean ± SEM in each group. n = 3/group.

3.1.2 Pup Survival in Different Febrile Seizure Dosage Groups

Mortality was measured in NS and S rat pups exposed to varying doses of LPS and KA by recording their survival rate. No deaths occurred in control groups. The overall survival rate of the pups was shown to be significantly dose-dependent ($F_{(4, 20)} = 3.583$; $p = 0.0233$). The NS-25% pups showed the highest survival rate, and a Dunn's multiple comparisons test showed that the NS-25% pups had a significantly higher survival rate than the NS-100% pups, (NS-25% vs NS-100% $p = 0.0151$). Data presented as mean ± SEM in Figure 3.2.

Preliminary Dose Test: Pup Survival Rate.

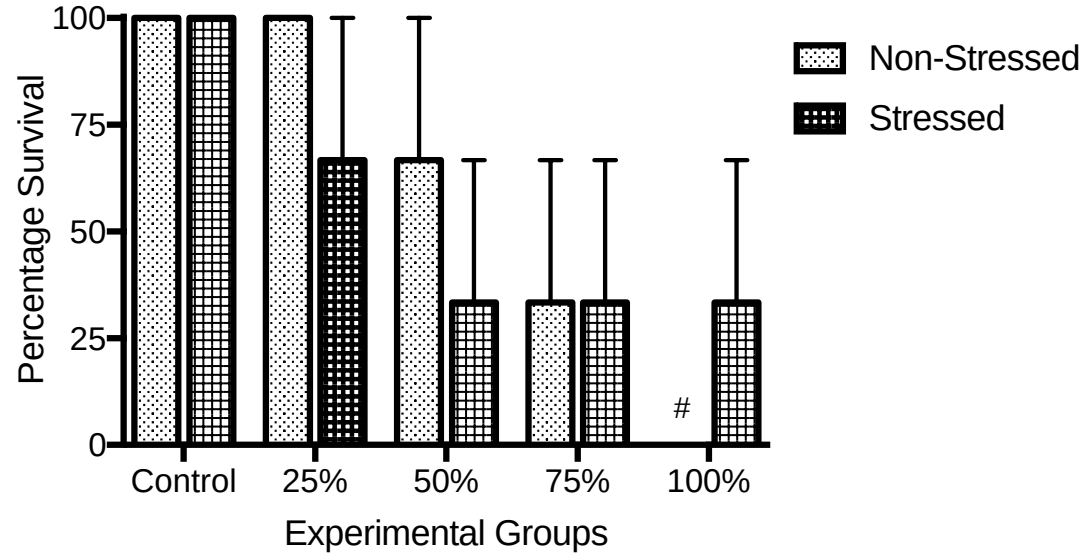

Figure 3.2 Study A: Graph depicting percentage survival for NS and S animals receiving different doses of LPS-KA. # $p < 0.05$ compared to NS-C & NS-25%. Data presented as mean ± SEM in each group. n = 3/group.

3.1.3 PROSTAGLANDIN E_2 (PGE_2) CONCENTRATIONS AT DIFFERENT DOSES OF LPS-KA

PGE_2 concentrations were measured in hypothalamic tissue of the surviving NS and S rat pups treated with the different doses of LPS and KA. The 100% dose was excluded due to the high mortality rate. Overall PGE_2 concentrations were significantly dose-dependent ($F_{(3, 14)} = 6.575$; $p = 0.0053$), most prominent within the NS pups, i.e. (NS-C vs NS-50% $p = 0.0128$), (NS-C vs NS-75% $p < 0.0001$). The lower 25% dose, was as effective as the higher 50% dose in inducing an upregulation in PGE_2 concentration in NS rats, but significantly less effective than the 75% dose i.e. (NS-25% vs NS-75% $p = 0.0047$), (S-25% vs S-75% $p = 0.0008$). Overall, a significant stress-induced decrease in PGE_2 concentrations was observed ($F_{(1, 14)} = 47.73$; $p < 0.0001$). There was also a significant interaction between stress and the dose of LPS-KA ($F_{(3, 14)} = 15.40$; $p < 0.0001$). Hypothalamic PGE_2 concentrations increased with increasing dose of LPS-KA in NS pups but decreased in S pups. Data presented as mean ± SEM in Figure 3.3

Preliminary Dose Test: PGE_2 Concentration.

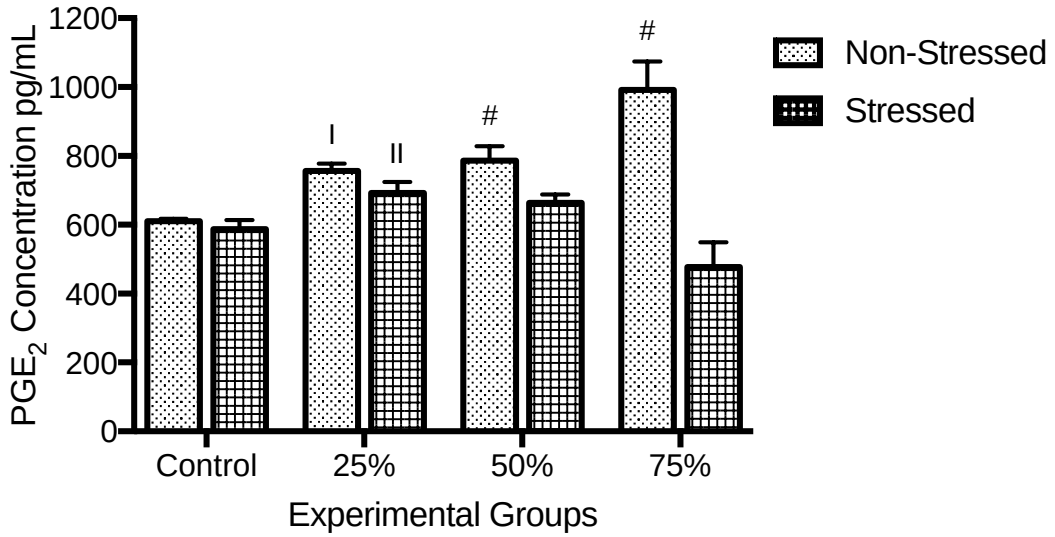

Figure 3.3 Study A: Graph depicting PGE_2 concentration for NS and S animals receiving different doses of LPS-KA. # $p < 0.05$ compared to NS-C. I $p < 0.05$ compared to NS-75%. II $p < 0.05$ compared to S-75%. Data presented as mean ± SEM in each group. n = 3/group, -1 animal for the S-50% and S-75%.

3.2 STUDY B: EFFECTS OF PRENATAL STRESS ON RAT MODELS OF FEBRILE SEIZURE

After selecting the appropriate dose of LPS-KA to induce febrile seizures, we determined the most efficient and effective febrile seizure rat model to use in our laboratory. NS and S PND 14 rat pups received either the selected 25% dose of LPS and KA, or they were exposed to a hyperthermic (H) environment to induce febrile seizures. The following 3 groups were assessed: (NS-C; S-C), (NS-LPS-KA; S-LPS-KA), and (NS-H; S-H). The average change in temperature, seizure severity and percentage survival rate for each group was analysed for statistical significance.

3.2.1 Assessment of Oral Temperature of Rat Models of Febrile Seizure

Fever progression was assessed by measuring oral temperature in NS and S rat pups exposed to either, LPS-KA or H. Both models elicited significant overall changes in oral temperature ($F_{(2, 36)} = 256.5$; $p < 0.0001$), with the NS-LPS-KA animals exhibiting a drop in oral temperature (NS-C vs NS-LPS-KA $p < 0.0001$), while H groups exhibited increased temperatures, i.e. (NS-C vs NS-H $p < 0.0001$), (S-C vs S-H $p < 0.0001$). Post hoc tests revealed a significant difference between the two models, i.e. (NS-LPS-KA vs. NS-H $p < 0.001$) and (S-LPS-KA vs. S-H $p < 0.001$). Both models were also significantly affected by stress ($F_{(1, 36)} = 28.28$; $p = 0.0001$), that was most evident in LPS-KA treated animals (NS-LPS-KA vs S-LPS-KA $p < 0.0001$). Data presented as mean ± SEM in Figure 3.4

Rat Models of Febrile Seizure: Change in Oral Temperature.

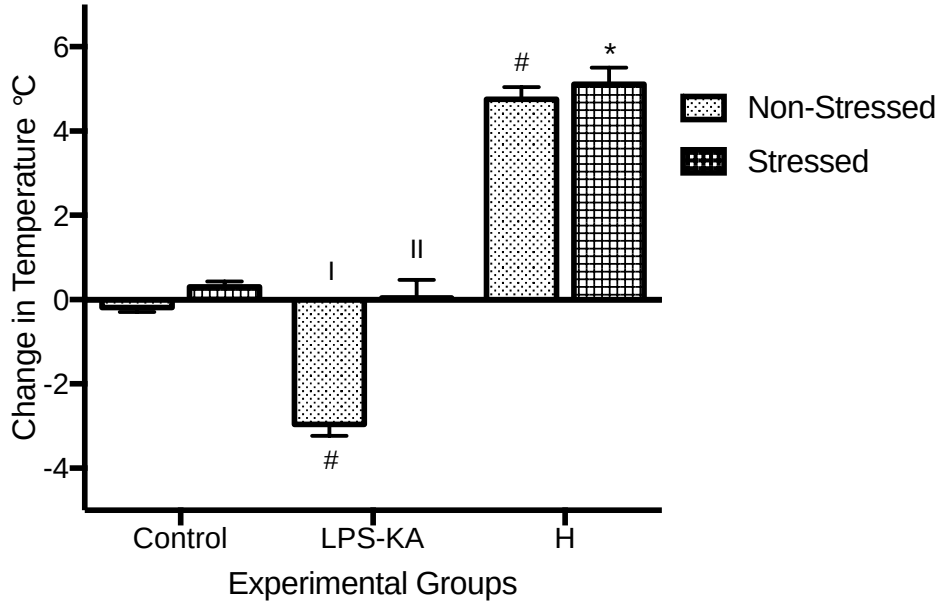

Figure 3.4 Study B: Graph depicting change in oral temperature for NS and S animals undergoing either LPS-KA or H induced febrile seizures. # $p < 0.05$ compared to NS-C. * $p < 0.05$ compared to S-C. I $p < 0.05$ compared to NS-H. II $p < 0.05$ compared to S-H. Data presented as mean ± SEM in each group. n = 7/group.

3.2.2 Assessment of Seizure Severity in Rat Models of Febrile Seizure

A Kruskal-Wallis and Dunn's multiple comparisons test showed both models were equally efficient in inducing seizures, i.e. the NS-LPS-KA pups experienced myoclonic jerks with many pups rearing and falling on their side, while the NS-H pups experienced mild myoclonic jerks with loss of postural control. In order to determine the effect of prenatal stress, the data of the pups in the seizure groups were analysed by two-way ANOVA, since the data were normally distributed. Prenatal stress significantly exacerbated seizure severity ($F_{(1, 24)} = 8.64$; $p = 0.0072$) for the two models, i.e. the S-LPS-KA pups experienced tonic-clonic convulsions with some pups falling on their side, while the S-H pups experienced clonic convulsions. Data presented as mean ± SEM in Figure 3.5

Rat Models of Febrile Seizure: Average Seizure Severity.

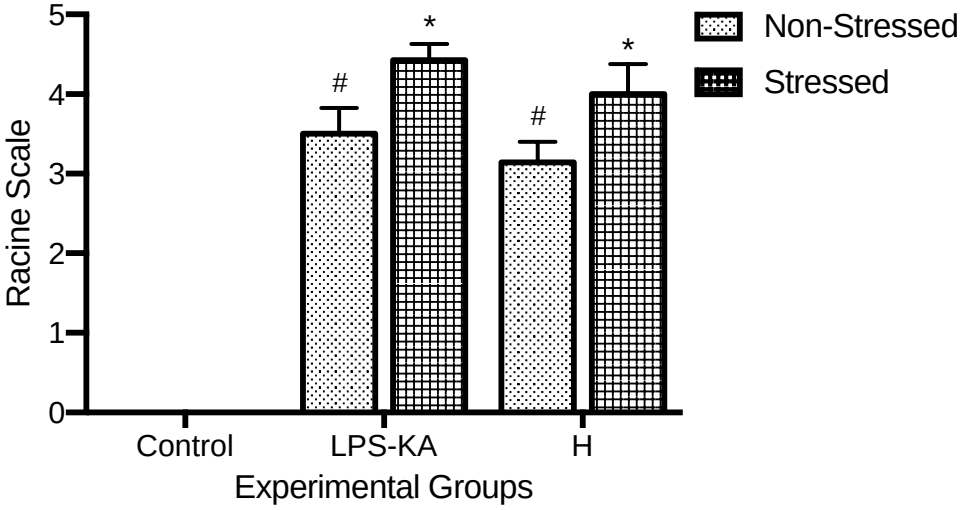

Figure 3.5 Study B: Graph of average seizure severity assessed for NS and S animals undergoing either LPS-KA or H induced febrile seizures. # $p < 0.05$ compared to NS-C. * $p<0.05$ compared to S-C. Data presented as mean ± SEM in each group. n = 7/group.

3.2.3 PUP SURVIVAL IN RAT MODELS OF FEBRILE SEIZURE

Mortality was measured in NS and S rat pups exposed to either LPS-KA or H to induce febrile seizures. No deaths occurred in NS and S pups for both the control and H groups. A significant overall effect of the model on the survival rate of the pups was observed ($p < 0.0047$). A Dunn's multiple comparison test showed S-LPS-KA pups had a significantly higher mortality when compared to the respective control, i.e. (S-C vs S-LPS-KA $p = 0.0322$), and S-H model, i.e. (S-LPS-KA vs S-H $p = 0.0322$). Data presented as mean ± SEM in Figure 3.6.

Rat Models of Febrile Seizure: Survival Rate.

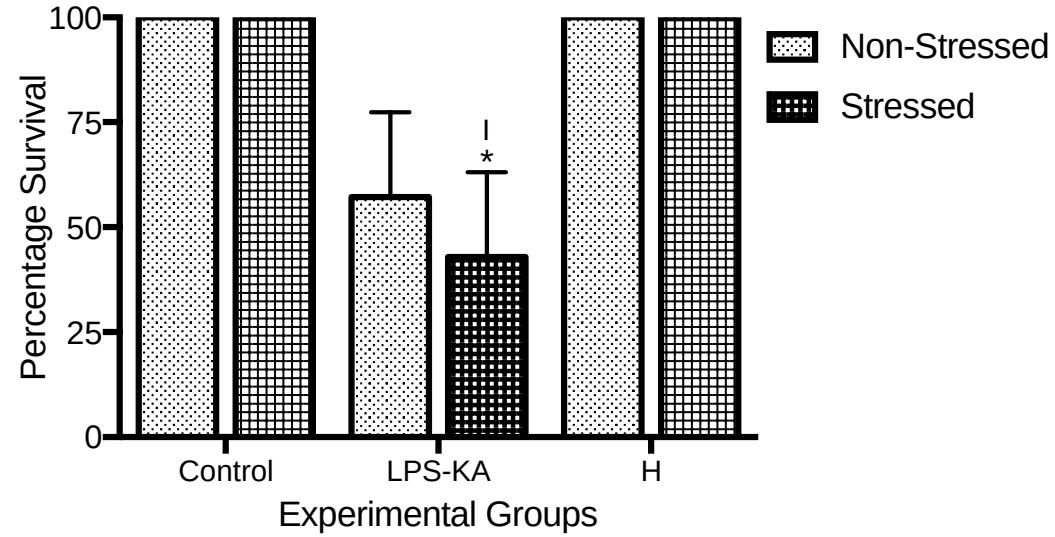

Figure 3.6 Study B: Graph depicting percentage survival rate assessed for NS and S animals undergoing either LPS-KA or H induced febrile seizures. * $p < 0.05$ compared to S-C. I $p < 0.05$ compared to S-H. Data presented as mean ± SEM in each group. n = 7/group.

3.2.4 PGE_2 AND RECEPTOR EP_3R CONCENTRATIONS BETWEEN RAT MODELS OF FEBRILE SEIZURE

PGE_2 concentrations and receptor EP_3R expression were measured in hypothalamic tissue of NS and S pups. A two-way ANOVA did not reveal any significant differences in PGE_2 concentrations, however a significant change was observed in the EP_3R gene expression. There was a significant effect of seizures ($F_{(2, 12)}$ = 2.369; p = 0.0498) which was mainly due to an increase in EP_3R expression in prenatally stressed seizure groups, however the overall effect of stress on EP_3R expression was not statistically significant ($F_{(1, 12)}$ = 3.731; p = 0.0774). Further analysis showed that S pups' PGE_2 concentrations correlated significantly with their EP_3R gene expression i.e. (S PGE_2 vs S EP_3R p = 0.0413; r = -0.998). Correlation between the NS pups PGE_2 and EP_3R were also seen, however this was not statistically significant i.e. (NS PGE_2 vs NS EP_3R p = 0.0709; r = -0.994. Data presented together as mean ± SEM in Figure 3.7.

Rat Models of Febrile Seizure: PGE_2 and EP_3R Concentration.

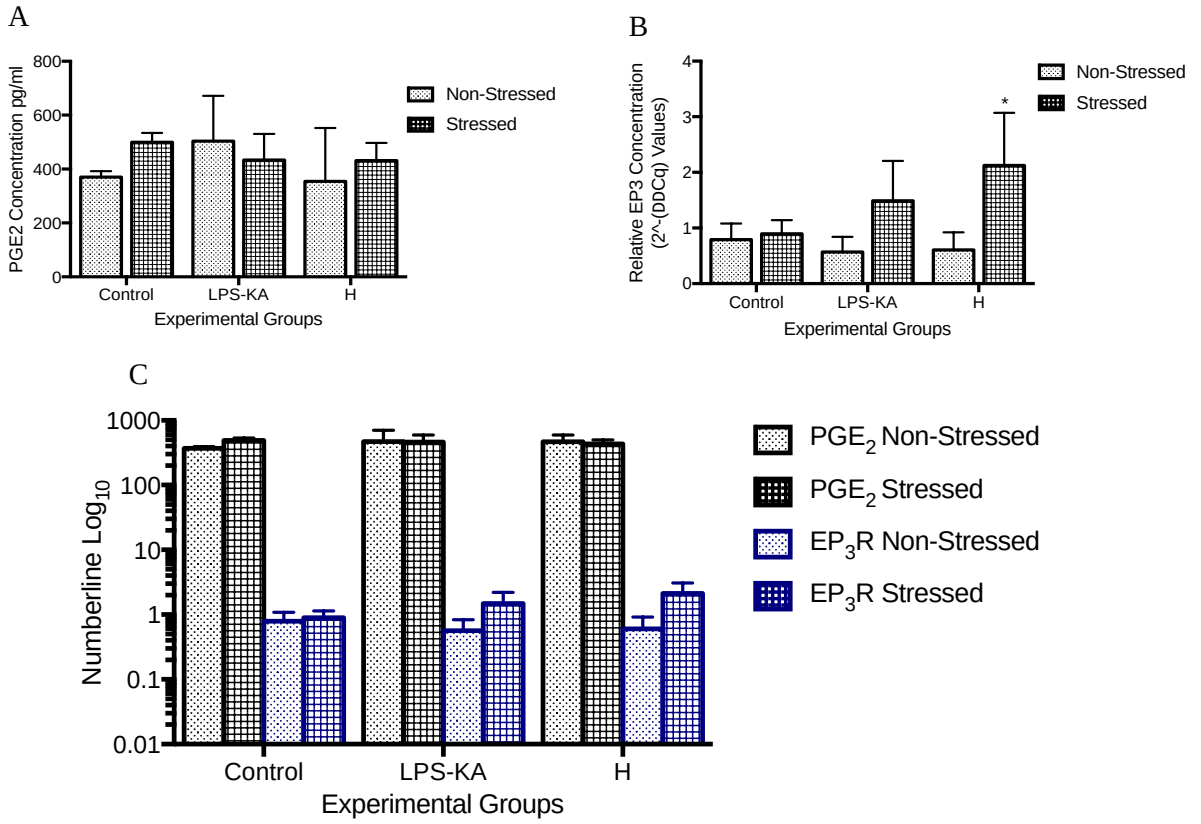

Figure 3.7 Study B: Graphs depicting hypothalamic PGE2 concentration and EP3R expression for NS and S pups undergoing LPS-KA or H induced febrile seizures. Graph A depicts PGE_2 concentration assessed via ELISA. Graph B depicts relative EP_3R concentration assessed via PCR. Graph C consists of both data sets against a Log10 scale. Data presented as mean ± SEM in each group. n = 3/group.

4 DISCUSSION

In this study, we re-examined our current febrile seizure rat model by assessing an alternative dose, as well as an alternative model to induce febrile seizure like symptoms in rat pups. The Heida et al. (2005) [26] lipopolysaccharide (LPS) and kainic acid (KA) model has successfully played a major role in inducing febrile seizures in our laboratory, however the dose has yielded a high mortality rate. Therefore, study A aimed to refine our current febrile seizure rat model by assessing an ideal LPS and KA (LPS-KA) dose derived from the original Heida et al. (2005) [26] dose. Study B aimed to determine the most effective and efficient rat model to mimic febrile seizures i.e. a modified LPS-KA model, or a hyperthermic environment (H) model.

Pregnant Sprague Dawley rats were separated into one of two groups in both studies, non-stressed (NS) where dams were left undisturbed throughout pregnancy, and stressed (S) where dams were exposed to restraint stress for 1 h a day for a total of 7 days in their third trimester. Febrile seizures were induced in the subsequent pups on postnatal day (PND) 14. Study A consisted of 5 groups that received different percentages of the original Heida et al. (2005) [26] dose including a control (C): (NS-C; S-C); (NS-25%; S-25%); (NS-50%; S-50%); (NS-75%; S-75%) (NS-100%; S-100%). Study B consisted of 3 groups including a C: (NS-C; S-C); (NS-LPS-KA; S-LPS-KA) (NS-H; S-H). All rat pups were sacrificed on PND 16 by means of a sharp guillotine to collect hypothalamic tissue to assess prostaglandin E_2 (PGE_2) and its receptor prostaglandin EP_3 receptor (EP_3R) concentrations.

4.1 STUDY A: PRELIMINARY DOSE OPTIMIZATION

Our findings for study A showed that all the doses resulted in febrile convulsions, however the higher NS-75% and NS-100% doses resulted in more severe stage 4 and 5 tonic-clonic convulsions which resulted in an average survival rate of 33% and 0% respectively. The lower NS-25% and NS-50% doses resulted in less severe seizures consisting of myoclonic jerks and animals falling on their sides, from which many pups recovered, thus experiencing a higher average survival rate of 100% and 67% respectively. The average survival rates of the pups were significantly dose dependant. Pups injected with higher doses of LPS-KA, experienced more severe seizures, which significantly correlated with a lower survival rate and greater drop in oral temperature. The temperature assessment showed an overall average dose-dependent decrease of 3.27 °C and 4.47 °C for the NS-75% and NS-100% doses and a 2.97 °C and 3.43 °C drop for the NS-25% and NS-50% doses respectively, as opposed to an increase despite keeping a consistent room temperature of 30 °C. This finding contradicted the previous study by Heida et al. (2005) [26] who found increased temperatures, but agreed with a study done by Krakauer et al. (2010) [50] who found a similar drop in body temperature when administering high doses of LPS [50]. Krakauer et al. (2010) [50] injected 7-10 week old male Balb/c mice with 80 µg/kg LPS, which resulted in hypothermia and a survival rate of only 52% [50]. Thus the possible

mechanism for the observed temperature drop may be due to the LPS, a gram-negative bacterial endotoxin that induces fever by triggering the synthesis and release of proinflammatory cytokines interleukin-1 beta (IL-1β), interleukin-6 and tumour-necrosis factor alpha by the innate immune system through toll-like receptors in the periphery (6, 35, 51-53). Hyperproduction of these proinflammatory cytokines has been shown to increase vascular permeability, and lead to an increase in proinflammatory cascades that may result in vascular collapse and ultimately systemic shock (54-56). We thus speculate that the hypothermia experienced in particular by our higher dose pups, was thus likely due to systemic toxic shock via the excessive production of these proinflammatory cytokines. This coincides with the fact that many pups that were injected with high doses of LPS-KA died. Pups injected with higher LPS-KA doses felt cool, stiff to the touch, and appeared sickly with decreased mobility further suggesting hypothermia and systemic shock.

In addition, the high mortality rate of the higher dose LPS-KA pups is thought to be due to extensive brain lesions, accompanied by neuronal loss and damage associated with the high doses of KA, a glutamate agonist that has been shown to increase intracellular calcium ion (Ca^{2+}) concentrations (29, 57-60). Glutamate is the predominant excitatory neurotransmitter of the adult mammalian brain (61, 62), but in excess, glutamate stimulates the influx of Ca^{2+} leading to its subsequent uptake by mitochondria, resulting in neuronal damage and eventual cell death, known as excitotoxicity (63-65). Under normal circumstances however, gamma-aminobutyric acid (GABA) controls glutamate transmission through a negative feedback mechanism by activating GABA receptors to regulate chloride and potassium ions, and decrease Ca^{2+} in order to modulate glutamate effects (6, 66-68). Excess IL-1ß produced during febrile seizures however has been shown to decrease levels of GABA receptor mediated currents, therefore further establishing a glutamate-GABA imbalance contributing to seizure generation and the high mortality rates (6, 44, 68, 69). High doses of LPS-KA thus result in a twofold excess production of proinflammatory cytokines, specifically IL-1β which triggers both fever producing pyrogens, and convulsion inducing neurotransmitter imbalances (5, 70-73).

Furthermore, our findings show that exposure to prenatal stress (S) during the third trimester exacerbated seizure progression in all the doses. The higher S-75% and S-100% doses resulted in more severe stage 5 repeated severe tonic-clonic convulsions, followed by a 2.63°C and 4.17°C drop in oral temperature respectively, leading to a lower survival rate of 33% for both doses. Although the lower S-25% and S-50% doses resulted in less severe seizures than the higher doses, they were significantly more severe than their NS counterparts, displaying myoclonic jerks and tonic convulsions with most animals falling on their sides, with a drop in temperature of 2.50°C and 3.10°C resulting in a survival rate of 67% and 33% respectively. These findings are in line with previous studies conducted in our laboratory by Qulu et al. (2012) (3), Cassim et al. (2015) (23) and Mkhize et al. (2017) (35) who found exacerbated stress responses.

The effect of prenatal stress on the brain of the offspring is well documented, as is the resultant dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (37, 74-76). Maternal malnutrition, stress, or trauma during pregnancy has been shown to result in dysregulation of the foetal HPA axis through downregulation of the protective enzyme 11beta-hydroxysteroid-dehydrogenase 2 in the maternal placental barrier, thus allowing increased entry and exposure of the foetus to maternal glucocorticoids (37). Excess cortisol (corticosterone in rats), exposure in utero is associated with increased activity of the offspring's HPA axis, and the now altered and over stimulated HPA axis often malfunctions resulting in higher than normal basal cortisol concentrations (37-39). Cortisol is important for the maturation of most foetal organ systems, but in excess it ultimately promotes the downregulation of its receptors (38-43). This dysregulation resulting in less cortisol uptake diminishes the immune system's capacity to respond to its anti-inflammatory actions, resulting in an increased proinflammatory response (43). This ultimately leaves the new-born more sensitive to future stress and more prone to infection than non-prenatally stressed neonates (38-43). This overstimulation of the HPA axis could explain why the S animals had exacerbated seizure severity, a greater drop in oral temperature and lower survival rates than their NS counterparts.

The neurochemical analyses supported behavioural findings by showing that NS-LPS-KA dosed pups experienced a dose-dependent increase in PGE_2 concentrations probably due to upregulated IL-1β levels associated with febrile seizure progression. Excess IL-1β has been shown to compromise the blood brain barrier, thereby crossing over into the central nervous system where it competitively binds to interleukin-1 receptor 1 and triggers cyclooxygenase-2 (COX-2) to catalyse the conversion of arachidonic acid into the pyretic prostaglandin PGE_2, that subsequently induces the preoptic nuclei of the hypothalamus to increase core temperature (5, 8-12, 71, 77). On the contrary however, prenatal stress lead to a decreased expression of PGE_2 in the S-LPS-KA pups which was particularly evident at high doses of LPS-KA. My previous unpublished work in our laboratory on LPS and KA treatment alone however showed prenatal stress increased the concentrations of PGE_2 in animals injected with only LPS, but that prenatal stress decreased PGE_2 concentrations in pups injected with only KA. We thus attribute the decreased PGE_2 concentrations of the S-LPS-KA animals to the KA treatment, and not the LPS. This finding contradicts previous work done by Kawaguchi et al. (2005) (15) who showed that PGE_2 levels were markedly increased 24 hs after KA injection (15). It has however been shown that if embryonic development is disturbed, such as during prenatal stress, PGE_2 concentrations may fall below normal base levels (78), thus suggesting a possible explanation for the decreased PGE_2 concentrations of the S-LPS-KA pups. The decreased PGE_2 concentrations could also likely be due to its relatively short half-life, whereas upregulation of its receptor expression takes place over a long time period (79). The lower PGE_2 concentration amongst S-LPS-KA pups could also be due to its numerous receptor subtypes that each would have increased their subsequent uptake of PGE_2 (80), thereby limiting freely available PGE_2.

Another point to consider is that PGE_2 is not catabolized in the brain of mammals, but rather in the lungs, kidney and the liver, and thus it has to be transported out of the brain tissue (79, 81), further limiting freely available PGE_2. A high enough brain-to-blood PGE_2 gradient has been shown to facilitate a net efflux of PGE_2 (79, 82, 83) as is experienced in animals after KA injection, particularly those that have also undergone prenatal stress, thus further upregulating PGE_2 efflux.

Although prenatal stress did not appear to affect the survival rate of the pups, it significantly affected seizure severity and the relative PGE_2 concentrations, which may have played a major role in the increased deaths of animals at the higher doses. Thus a 25% dose of 50 µg/kg LPS and 0.44 mg/kg KA was selected to induce febrile seizures as this proved sufficient to induce seizures while maintaining a relatively high survival rate. The 25% dose significantly decreased the drop in temperature, seizure severity and duration, and resulted in a higher survival rate.

4.2 STUDY B: RAT MODELS OF FEBRILE SEIZURE STUDY

Our findings for study B showed that both the modified LPS-KA and H models were successful in inducing febrile seizures. Animals treated with LPS-KA developed significantly more severe seizures than the H animals, and in both models, prenatal stress significantly exacerbated seizure severity, with the S-LPS-KA and S-H animals experiencing more severe seizures than their NS counterparts. The NS-LPS-KA pups experienced moderate stage 3 and 4 seizures consisting of myoclonic jerks and clonic convulsions with many falling over onto their sides, while the S-LPS-KA pups experienced significantly more severe stage 4 and 5 seizures consisting of tonic-clonic convulsions with almost all animals falling over. The NS-LPS-KA pups showed a decrease of 2.99°C in oral temperature, and although this behavioural result again contradicted Heida et al. (2005) (26), it agreed with the study by Krakauer et al. (2010) (50) who found that LPS caused hypothermia (50). As previously discussed, the proposed mechanism for the decrease in oral temperature may be due to excessive production of proinflammatory mediators that lead to toxic systemic shock (54-56). The S-LPS-KA pups exhibited a slight increase in temperature, which coincides with the norm for LPS injection. We believe that the S-LPS-KA pups exhibited an increased temperature compared to their NS counterparts due to an exacerbated stress-induced response to LPS. This lead to an exacerbated production of IL-1ß known to induce fever by triggering COX-2 to catalyses the conversion of arachidonic acid into PGE_2 (11), which left the S animals more prone to developing a fever than NS counterparts to the same stressor (8, 38). The neurochemical results supported the behavioural findings by showing an increased concentration in both PGE_2 and EP_3R for the NS-LPS-KA pups, but as before a decrease in PGE_2 concentration for the S-LPS-KA pups. Interestingly, the S-LPS-KA pups expressed a greater increase in its receptor EP_3R concentrations than the NS-LPS-KA pups. The greater increase in EP_3R expression suggests an initially greater increase in PGE_2 concentration, while the recorded decrease in PGE_2 concentration again suggests an exacerbated stress effect leading to a high brain-to-blood gradient, resulting in its net efflux out of the brain tissue as previously discussed (79, 82, 83).

The H model produced 100% seizure success, with 100% survival. The NS-H pups experienced mild to moderate stage 2 and 3 seizures consisting of facial automations, loss of posture and myoclonic jerks, while the S-H pups experienced more severe stage 4 seizures consisting of myoclonic jerks and clonic convulsions. NS-H and S-H pups experienced a significant rise in oral temperature of 4.76°C and 5.11°C respectively, which are symptoms associated with fever, however our neurochemical analysis did not support this finding with regards to increased PGE_2 concentrations. Although prenatal stress exacerbated seizures, it lead to a decrease in PGE_2 concentrations. Prenatal stress did however lead to an increase in the receptor EP_3R expression, which we again attribute to an initially increased production of PGE_2 through upregulated IL-1ß stimulation, after which an overall net efflux of excess PGE_2 out of the brain occurred (79, 82, 83). This would explain the decreased PGE_2 concentration and increased EP_3R expression at the time of decapitation for the S-H pups compared to the NS-H pups. Although the H model was successful in inducing behavioural seizures, we postulate it was due to a different mechanism than that of the LPS-KA infection model. Elevated body temperature due to hyperthermia is known to result in an increased rate of breathing known as hyperventilation, which is known to raise pH levels resulting in neuronal excitability (84-87).

Based on these findings, we thus suggest the use of the modified LPS-KA model for future studies of the mechanism of infection-induced fever resulting in convulsions. This model accurately mimics an infection that predisposes children to febrile seizures, as well as the predisposition to future complications such as temporal lobe epilepsy. If one needs merely to mimic a fever in the absence of infection, followed by convulsions, we suggest the H model due to it being less invasive, and ensuring a higher survival rate. We do recommend however that both models be further studied for a more thorough understanding of the mechanisms involved.

5 CONCLUSION

Animal models are vital tools in understanding the mechanism that potentiate febrile seizures, as well as numerous other human conditions, and are essential in developing precautionary and therapeutic strategies, and in the preclinical development of drugs to combat these conditions. Due to the diverse genetic makeup of children, and the presence of many uncontrollable external factors, i.e. prenatal stress, it would be impossible to effectively demonstrate the complete mechanisms and consequences related to febrile seizures if not for animal models. By refining the Heida et al. (2005) (26) LPS-KA model, we achieved 100% febrile seizure success, while significantly reducing mortality rates. These simple, non-reoccurring, benign seizures more accurately represent the simple febrile seizures most often experienced by otherwise healthy young children.

6 AUTHOR CONTRIBUTIONS

YM and LQ designed the experiments, and jointly developed the structure and arguments for the paper. YM ran all experiments and analysed all data under the supervision of LQ, and both authors agree with the manuscript results and conclusions.

7 CONFLICT OF INTERESTS

The authors declare no personal or financial conflicting interests.

8 ACKNOWLEDGMENTS

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10 APPENDICES

10.1 APPENDIX A

Gmail Yasmin Malik <y6malik@gmail.com>

Submission Confirmation
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10.2 APPENDIX B

JOURNAL OF NEUROSCIENCE METHODS

Submissions Being Processed for Author Yasmin Malik, Hons

Page: 1 of 1 (1 total submissions) Display 10 results per page.

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
Action Links	JNEUMETH-D-19-00389	The Effects of Prenatal Stress on Hypothalamic PGE2 and its Receptor Concentrations in Rat Models of Febrile Seizure	01 Jul 2019	01 Jul 2019	With Editor

Page: 1 of 1 (1 total submissions) Display 10 results per page.

<< Author Main Menu

10.3 APPENDIX C

Study A: Preliminary Toxicity Test Behavioural Results. Table containing average data for change in oral temperature, seizure severity and survival rate.

	Non-Stressed			Stressed		
	Change in oral Temperature	Average Seizure Severity	Average Survival percentage	Change in oral Temperature	Average Seizure Severity	Average Survival percentage
Control	-0.73°C	0.00	100.00%	-0.60°C	0.00	100.00%
25%	-2.97°C	2.33	100.00%	-2.50°C	3.33	83.30%
50%	-3.43°C	3.33	83.33%	-3.10°C	3.67	66.58%
75%	-3.27°C	4.00	66.67%	-2.63°C	4.67	22.10%
100%	-4.47°C	4.67	7.00%	-4.17°C	5.00	29.15%

Study A: Preliminary Toxicity Test Behavioural Results. Change in Oral Temperature, Seizure Severity and Survival. Initial and post treatment oral temperature was measured for each pup, thereafter seizers were scored 0 – no response, to 5 – repeated severe tonic-clonic convulsions on a Racine scale. Survival was regarded as surviving until sacrifice. n = 3/group.

10.4 APPENDIX D

Study B: Febrile Seizure Rat Model Comparison: Behavioural Results. Table containing average data for change in oral temperature, seizure severity and survival rate.

	Non-Stressed			Stressed		
	Change in oral Temperature	Average Seizure Severity	Average Survival percentage	Change in oral Temperature	Average Seizure Severity	Average Survival percentage
Control	-0.19°C	0.00	100.00%	-0.17°C	0.00	100.00%
LPS-KA	-2.99°C	3.50	76.00%	+0.04°C	4.43	55.00%
H	+4.76°C	3.14	100.00%	+5.11°C	4.00	100.00%

Study B: Febrile Seizure Rat Model Comparison: Behavioural Results. Change in Oral Temperature, Seizure Severity and Survival. Initial and post treatment oral temperature was measured for each pup, thereafter seizers were scored 0 – no response, to 5 – repeated severe tonic-clonic convulsions on a Racine scale. Survival was regarded as surviving until sacrifice. n = 7/group.

10.5 APPENDIX E

Figure 3.1 Study A: Preliminary Toxicity Test Behavioural Data Significance. Oral temperature and seizure severity for the toxicity study was assessed for NS and S animals receiving different doses of LPS and KA.

Oral Temperature

NS-C vs NS-25% p=0.0360
NS-C vs NS-50% p=0.0063
NS-C vs NS-75% p=0.0116
NS-C vs NS-100% p=0.0001
S-C vs S-50% p=0.0130
S-C vs S-100% p=0.0003

Seizure Severity

NS-C vs NS-25% p=0.0014
NS-C vs NS-50% p<0.0001
NS-C vs NS-75% p<0.0001
NS-C vs NS-100% p<0.0001
S-C vs S-25% p<0.0001
S-C vs S-50% p<0.0001
S-C vs S-75% p<0.0001
S-C vs S-100% p<0.0001
NS-25% vs NS-75% p=0.0342
NS-25% vs NS-100% p=0.0014
S-25% vs S-100% p=0.0342

Figure 3.2 Study A: Preliminary Toxicity Test Survival Rate Significance. The survival rate was assessed for NS and S animals receiving different doses of LPS and KA.

NS-C vs NS-100% p=0.0151
NS-25% vs NS-100% p=0.0151

Figure 3.3 Study A: Preliminary Toxicity Test PGE2 Concentration. The concentration of PGE2 was assessed for NS and S animals receiving different doses of LPS-KA.

NS-C vs NS-25% p=0.0344
NS-C vs NS-50% p=0.0128
NS-C vs NS-75% p<0.0001
NS-25% vs NS-75% p=0.0047
NS-50% vs NS-75% p=0.0131
NS-50% vs S-50% p=0.0131
S-C vs S-75% p=0.0027
S-25% vs S-75% p=0.0008
S-50% vs S-75% p=0.003
S-75% vs S-100% p=0.048

Figure 3.4 Study B: Febrile Seizure Rat Model Comparison: Change in Oral Temperature significance. Data assessed for NS and S animals undergoing either LPS-KA or H induced febrile seizures

NS-C vs NS-LPS-KA p<0.0001
NS-C vs NS-H p<0.0001
S-C vs S-H p<0.0001
NS-LPS-KA vs NS-H p<0.0001
NS-LPS-KA vs S-LPS-KA p<0.0001
S-LPS-KA vs S-H p<0.0001

Figure 3.5 Study B: Febrile Seizure Rat Model Comparison: Average Seizure Severity significance. Data assessed for NS and S animals undergoing either LPS-KA or H induced febrile seizures.

NS-C vs NS-LPS-KA p=0.0416

NS-C vs NS-H p=0.0143

S-C vs S-LPS-KA p=0.0003

S-C vs S-H p=0.0052

Figure 3.6 Study B: Febrile Seizure Rat Model Comparison: Significant Survival Rates. Data assessed for NS and S animals undergoing either LPS-KA or H induced febrile seizures.

S-C vs S-LPS-KA p=0.0322

S-LPS-KA vs S-H p=0.0322

Figure 3.7 Study B: Febrile Seizure Rat Model Comparison: Significant PGE2 and EP3R correlation. Data assessed for NS and S animals undergoing either LPS-KA or H induced febrile seizures.

NS-H EP_3R vs S-H EP_3R p=0.0153

S-C EP_3R vs S-H EP_3R p=0.0219

NS-H PGE_2 vs NS-H EP_3R p=0.0394

S-C PGE_2 vs S-C EP_3R p=0.0272

NS-LPS-KA PGE_2 vs NS-LPS-KA EP_3R p=0.0363

S-LPS-KA PGE_2 vs S-LPS-KA EP_3R p=0.0423

CHAPTER THREE

Chapter three consists of a synthesis followed by a recommendations section from my personal experience with this study.

SYNTHESIS

Febrile seizures are the most common type of seizures experienced by infants and young children between the ages of 3 months to 6 years. Febrile seizures are often triggered by a fever, usually occurring after the onset of a systemic infection. Southern African has shown a growing prevalence of febrile seizures due to numerous risk factors, i.e. inadequate medical facilities and malnutrition. A seizure is defined as sudden, and uncontrolled electrical firing of neurons that may translate into uncontrolled convulsions of the body, and or altered consciousness. Seizures that result in convulsions are usually tonic clonic seizures and involve rhythmical jerking movement of one's body, or part thereof. Tonic seizures occur when the muscles suddenly become stiff, and atonic seizures occur when the muscles suddenly become relaxed. Both tonic and atonic seizures are brief, and don't induce convulsions, but often result in the person falling over. Absence seizures result in a moment of blank unresponsiveness. The patient is often unaware that they are having an absence seizure, as there are no physical signs of convulsing or change in muscle tension.

Febrile seizures occur with the activation of the immune system to release proinflammatory cytokines which subsequently induce a fever. The increased release of these cytokines lead to a neurotransmitter imbalance in the brain, causing neuronal hyper-excitability leading to convulsions. The immune system is the body's defence line that protects it from invading pathogens via eliciting an inflammatory response to fight and remove these invading pathogens. During a febrile seizure, there is an overactivation and release of proinflammatory cytokines that compromise the protective blood brain barrier, thus allowing these proinflammatory cytokines to enter the brain. Proinflammatory cytokines are small protein molecules that are released from immune system cells to convoy a chemical message to initiate an inflammatory response. Increased proinflammatory cytokines in the brain, particularly in the preoptic area of the hypothalamus, over stimulate neuronal cells and enzymes, such as cyclooxygenase-2, leading to an increase in core body temperature, resulting in fever. Enzymes are another type of protein molecule, but these act as catalysts for chemical reactions, rather than just messengers. The enzyme cyclooxygenase-2 in particular catalyses the production of prostaglandin E_2. Prostaglandins are lipid metabolites that play a major role in inflammation and pain in the body. Fever is triggered in the preoptic area of the hypothalamus by prostaglandin E2 binding prostaglandin 3 receptors to inhibit preoptic warm-sensitive neuron signals, thus preventing heat loss, and increasing cold-sensitive neuron signals, thus enhancing heat production and retention. The hypothalamus is split into 4 main regions, each responsible for a number of functions from regulating body temperature, sleep and appetite, to hormone release and emotional controls. The preoptic nuclei is found within the rostral most part of the hypothalamus, and is most often referred to as the thermoregulatory hub as well as the fever centre. Excess proinflammatory cytokines in the brain also lead to other complications such as neurotransmitter imbalances, resulting in unstable membrane potentials, and neuronal death due to cell overactivation.

In order to investigate the potential consequences of febrile seizures, the human condition was mimicked in Sprague-Dawley rats via a two-step method to induce fever, and then convulsions. A preliminary toxicity study was carried out to determine the ideal dosage of drugs to use in order to induce febrile seizures, while maintaining a relatively high survival rate. To induce an infection like reaction, rats were injected with lipopolysaccharide (LPS) the major component of the outer wall membrane of Gram-negative bacteria. LPS induces a strong defensive response from animal immune systems, by triggering enzymes that catalyses the processes leading to a fever like situation. Kainic acid (KA) is an excitotoxic and convulsive neurochemical that causes generalized tonic-clonic convulsions, and was thus used to mimic the convulsion experienced during a febrile seizure. KA was injected 2 h 30 min after the initial LPS injection to allow for the rise in temperature. This model of mimicking febrile seizes has been invaluable in studying the causes and progression of the disorder, but it has an extremely high mortality rate. We thus refined the model by reducing the dose of LPS and KA injected to 25% of the original Heida et al. (2005) model dose. By reducing the dose for the model, we were able to effectively mimic febrile seizures, while maintain a high survival rate.

Aside from refining the Heida et al. (2005) model, we also looked into introducing a new rat model of febrile seizure to our laboratory. This new model was based on previous work done by Jiang et al. (1999) and Yagoubi et al. (2015) who showed that extended exposure to a hyperthermic environment can induce febrile seizures by increasing core body temperatures beyond threshold temperatures required to evoke febrile seizures. These heat induced seizures are often more subtle than those induced by chemoconvulsants, and thus we aimed to refine and introduce the model to our laboratory as a less invasive method for study febrile seizures. The model was effective in mimicking seizures, and maintaining a high survival rate, however it was inefficient in correctly mimicking fever progression that occurs before a febrile seizure onset as a result of infection.

Our work, as with previous work done in our laboratory by Qulu et al. (2012), Cassim et al. (2015) and Mkhize et al. (2017) showed that exposure to prenatal stress during the third trimester exacerbated febrile seizures in rat offspring. Prenatal stress accounts for all types of stress experienced by pregnant mothers, with the most prominent effects occurring during the last trimester. In order to assess the effects of prenatal stress in our study, we subjected half of our pregnant dams to restraint stress during the third trimester. This consisted of placing the pregnant dams in rodent restrainers for 1h a day for a total of 7 days. The pups from the stressed dams then underwent the same procedures as the non-stressed pups to induce febrile seizures. Prenatal stress lead to greater changes in temperature, more severe seizures, and longer recovery times. Prenatal stress however did not seem to effect the upregulation of prostaglandins responsible for fever generation, however it did lead to significantly increased prostaglandin receptors concentrations.

RECOMMENDATIONS

One thing to be learnt from this study is the need to religiously preform trial runs before commencing with the main study. Each animal model used should always bear in mind the 3 R's, described by nc3Rs.org., i.e. Replacement, Reduction and Refinement. Where replacement is impossible, as such with total body mechanism studies, animals should be reduce by properly refining all experiments.

The LPS-KA model has long been used in research laboratories all around the world, and has proved invaluable in understanding the pathophysiology of febrile seizures. Through iteration, we managed to overcome some of its shortfalls and have thus made a list of suggestion for this model.

- Reduce the dose of LPS and KA if possible.
- LPS and KA should always be made at most 3 days before injections, and stored frozen in aliquots in air tight glass containers, protected from light.
- LPS and KA, as with all drugs should always be administered at room temperature. Allow the solution to thaw and then reach room temperature after removing from the fridge/freezer.
- Placing the rat pups in a small container on ice for a few minutes' assists in increasing body temperature naturally. Rat pups should not be kept on ice for an extended period of time.

The hyperthermic model proved to be useful in inducing seizures, and maintaining survival, however gene analysis showed that the pyrogenic prostaglandin and its receptor were not fully stimulated, as is found with fever occurring naturally due to an underlying infection. An injection of LPS prior to heat treatment could prove quite useful in inducing cytokine release, along with an increased core body temperature. This could result in more realistic febrile seizures, without the excessive brain cell damage caused by injection of KA.

Given an unlimited budget, it would have been ideal to run multiple gene analysis test. Testing for interleukin-beta (IL-1ß), interleukin-1receptor antagonist (IL-1ra), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNFα), corticosterone, cyclo-oxygenase-2 (COX-2) and numerous prostaglandin by-products would have given a better overview. Other than brain hypothalamic tissue, liver and kidney tissue should also be analysed. We attempted to collect trunk blood, but our animals were too small and thus sufficient volumes of blood were not obtained.

Animal models of febrile seizures can also be translated into lower class non-mammalian animals such as the fruit fly, the zebrafish, and the worm (*nematode Caenorhabditis elegans*). These models could prove to be more time and cost effective.

A final recommendation would be the implementation of an automated scoring software programme, this would limit bias of visual scoring thereby standardizing scoring parameters.

CHAPTER FOUR

Chapter four contains all relevant appendices for the thesis, including the ethics certificate and in-depth descriptions on the methods used for the ELISA and PCR assays.

APPENDICES

APPENDIX A: ETHICAL CLEARANCE CERTIFICATE

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

14 August 2018

Ms Yasmin Malik (217032991)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Ms Malik,

Protocol reference number: AREC/045/018M
Project title: The effects of prenatal stress on the Preoptic Nuclei of Febrile Seizure Rat Models

Full Approval – Research Application

With regards to your revised application received on 22 June 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS:

- The Animal Facility must be informed immediately the number as soon as the pups are born. The pups that are not being used for the study must be returned to the animal facility after weaning, with their parents.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 14 August 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Lihle Qulu
Cc Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Anita Engelbrecht
Cc BRU – Dr Linda Bester

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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APPENDIX B: CONFERENCE OUTPUT

Oral Presentation:

1st SANS UKZN Neuroscience Symposium 2018

Neuroinflammation

Nelson R Mandela School of Medicine, K-RITH Building

8 June 2018

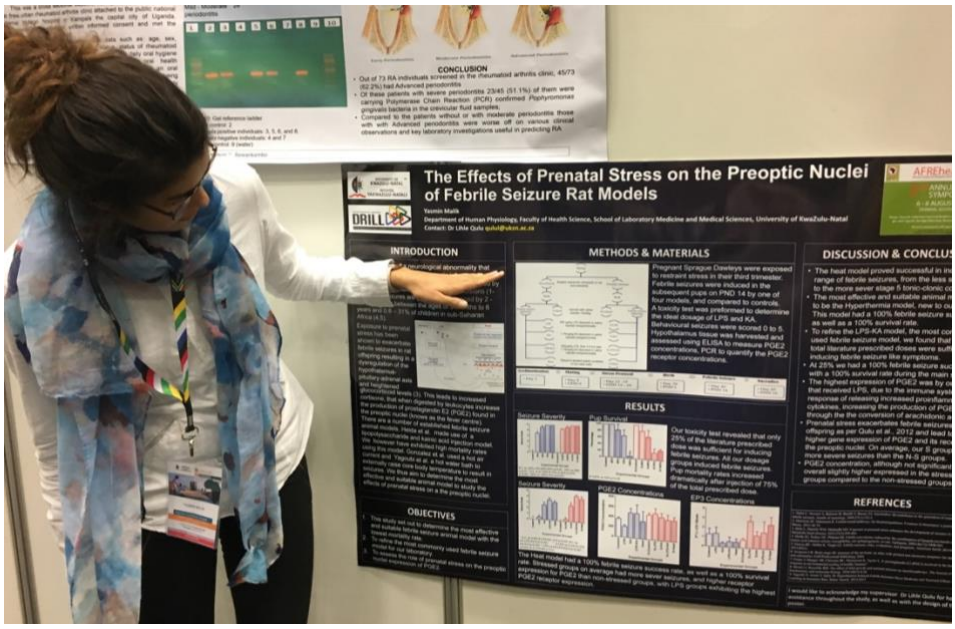
Poster Presentation:

2nd AFREhealth Symposium 2018

Towards Achieving Universal Health Coverage in Africa – Creating Synergies and Capacity through Education, Research and Quality Health Services

Durban International Convention Centre

6 – 8 August 2018



Upcoming Oral Presentation:

2nd SANS UKZN Neuroscience Symposium 2019

Effect of Early Life Trauma on The Brain

Nelson R Mandela School of Medicine, K-RITH Building

5 July 2019

APPENDIX C

Full description of the dose study postnatal handling of the different groups.

PRELIMINARY TOXICITY STUDY

On postnatal day (PND) 14, pups were removed from their dams and separated into five study groups including a control. Each group of animals received a different percentage of the original Heida et al. (2005) (1) dose of both lipopolysaccharide (LPS) and kainic acid (KA) to induce febrile seizures. All injections were preformed intraperitoneally (i.p.) using a 29G 0.5mm insulin needle.

CONTROL(C)

The control groups, NS-C and S-C were injected twice with 10 ml/kg of plain saline solution 2.5 hours apart to compensate for any additional stress experienced by the injections in the experimental groups.

GROUP 25%

This group received 25% of the literature prescribed 200 µg/kg LPS and 25% of the 1.75 mg/kg KA prescribed dose (1-3). Pups in this group were injected with 50 µg/kg LPS dissolved in 10 ml/kg saline followed by a second injection 2.5 hours later of 0.44 mg/kg KA dissolved in 10 ml/kg saline.

GROUP 50%

This group received 50% of the literature prescribed 200 µg/kg LPS and 25% of the 1.75 mg/kg KA prescribed dose (1-3). Pups in this group were injected with 100 µg/kg LPS dissolved in 10 ml/kg saline followed by a second injection 2.5 hours later of 0.88 mg/kg KA dissolved in 10 ml/kg saline.

GROUP 75%

This group received 75% of the literature prescribed 200 µg/kg LPS and 25% of the 1.75 mg/kg KA prescribed dose (1-3). Pups in this group were injected with 150 µg/kg LPS dissolved in 10 ml/kg saline followed by a second injection 2.5 hours later of 1.31 mg/kg KA dissolved in 10 ml/kg saline.

GROUP 100%

Pups in this group received the full literature prescribed dose, i.e. 200 µg/kg LPS dissolved in 10 ml/kg saline followed 2.5 hours later by 1.75 mg/kg KA dissolved in 10ml/kg saline (1-3).

Full description of the model study postnatal handling of the different groups.

POSTNATAL HANDLING AND FEBRILE SEIZURE INDUCTION

On postnatal day (PND) 14 both the stressed (S) and the non-stressed (NS) rat pups were removed from their dams and separated into four main febrile seizure induction study groups, and a control (C) group. All injections were preformed intraperitoneally (i.p.) using a 29G 0.5mm insulin needle.

CONTROL (C)

The control groups, NS-C and S-C were injected twice with 10 ml/kg of plain saline solution 2.5 hours apart to compensate for any additional stress experienced by the second injection in the experimental groups.

LIPOPOLYSACCHARIDE AND KAINIC ACID (LPS-KA) GROUP

The pups in this group were injected with the preselected dose (25%) of the original Heida et al. (2005)(1) model. The pups in this group, NS-LPS-KA and S-LPS-KA were injected i.p. with 50µg/kg of LPS dissolved in saline 10ml/kg, followed by a second i.p. injection 2.5 hours later of 0.4375 mg/kg KA dissolved in saline 10ml/kg.

HEAT (H) INDUCED GROUP

The heat induced groups NS-H and S-H, were exposed to heat to induce a fever like situation . To induce hyperthermia, pups were placed into clear plastic containers with perforated upper sides and lids to allow for sufficient ventilation. The container floor was lined with polystyrene to prevent the pups feet from burning, and was changed between each trial to minimise additional stress. The container was submerged into a water bath warmed at 55-60°C. The rats were kept in this hyperthermic situation for about 20 min to allow for a rise in their core temperature. Once a myoclonic jerk was experienced, the pup was removed and transferred back to room temperature where the febrile seizure was assessed. The pup was cooled, rehydrated, and dried off before being returned to its home cage.

References:

1. Heida JG, Teskey GC, Pittman QJ. Febrile convulsions induced by the combination of lipopolysaccharide and low-dose kainic acid enhance seizure susceptibility, not epileptogenesis, in rats. Epilepsia. 2005;46(12):1898-1905.
2. Qulu L, Daniels WM, Mabandla MV. Exposure to prenatal stress enhances the development of seizures in young rats. Metab Brain Dis. 2012;27(3):399-404.
3. Cassim S, Qulu L, Mabandla MV. Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats. Neurobiology of learning and memory. 2015;125:195-201.

APPENDIX D

Full description of the ELISA protocol followed for this study.

COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Hypothalamic tissue collected from the dose study animals as well as from the febrile seizure model comparison study and assessed using ELISA to detect the concentration of prostaglandin E2 (PGE2) concentrations.

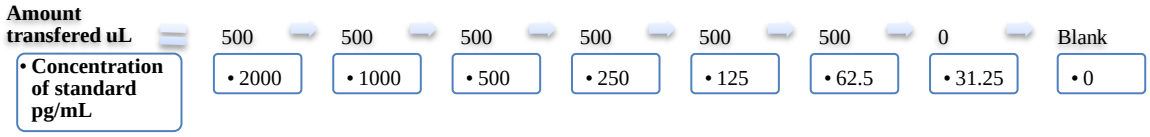
The Elabscience PGE2 (Prostaglandin E2) ELISA Kits (E-EL-0034) (Elabscience, Wuhan, China) were used for both studies, and thus the procedure for the pre-study and the main study were the same. The overall ELISA procedure is comprised of three main tasks, sample collection, assay procedure, and then determining the optical density. The Wash Buffer was added to a BioTek ELx50 plate washer (BioTek, Highland Park, USA) to aspirate and wash the plate. The plate was incubated in an oven at 37°C. The optical density was read at 450nm in a SPECTROstar Nana absorbance plate reader. (BMG, Labtech Ortenberg, Germany).

The ELISA reagents were prepared according to the manufactures manual, shown below the reference standard diluted in serial from the supplied 2000pg/mL down to 31.25 as shown below.

ELISA reagent preparation

Reagent	Diluent	Dilution ratio	Time before use
Concentrated wash buffer	Distilled water	1:25	
Biotinylated detection Ab working solution	Biotinylated detection Ab diluent	1:100	15 min
Concentrated HRP conjugate	Concentrated HRP diluent	1:100	15 min
Reference standard	Reference standard and sample diluent	2:1 serial dilution	

Elisa standard serial dilution



To prepare the samples to be homogenised, the collected frozen tissue was allowed to thaw on ice, before being diluted in phosphate-buffered saline (PBS) in a 1:9 ratio. All samples were homogenised using a sonicator (Qsonica, MODEL CML-4) on ice in their original collection Eppendorf's, and then centrifuged (Hermle LASEC, Germany) at 5000 x g and the supernatant was collected for the assay procedure.

ASSAY PROCEDURE:

- A volume of 50 µL of each standard was added in duplicate side by side into the first two columns of the provided 96-well plate, followed by the samples applied in the same manner.
- An equal volume of the diluted Biotinylated Detection Ab solution was immediately added to each well. The plate was then sealed and incubated for 45 min.
- A wash machine (BioTek, Highland Park USA) was used to aspirate, soak and wash the plate 3 times.
- After washing, 100 µL of the diluted HRP Conjugate working solution was added to each well, then the plate was sealed and incubated for 30 min.
- The plate was returned to the wash machine (BioTek, Highland Park USA) and washed 5 times.
- After washing, 90 µL of the Substrate Reagent was added to each well, the plate sealed and incubated for just short of 15 min.
- Finally, 50 µL of Stop Solution was added to each well in the same order as the Substrate Reagent.

The plate was immediately taken to the SPECTROstar Nana (BMG, Labtech Ortenberg, Germany) absorbance plate reader to determine optical density at 450nm.

(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !)

PGE2(Prostaglandin E2) ELISA Kit

Synonyms: PG-E2, Dinoprostone

Catalog No : E-EL-0034
96T

This manual must be read attentively and completely before using this product .

If you have any problems, please contact our Technical Service Center for help.

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

APPENDIX E

Full description of the PCR protocol followed for this study.

REAL-TIME POLYMERASE CHAIN REACTION (PCR)

Real time PCR was employed to quantify the expression of prostaglandin EP3 receptor (EP3R) levels in hypothalamic tissue collected from the model study animals. The overall polymerase chain reaction (PCR) procedure is comprised of three main tasks, isolating the RNA from the tissue samples, converting the isolated RNA into complementary DNA (cDNA), and then amplifying the target DNA section.

RNA ISOLATION

The Zymo Research Quick-RNA™ Miniprep Kit (ZAR1054) kit was used to isolate RNA from our tissue samples. The three step protocol from the manufactures manual was followed, which included homogenization, clearing and gDNA removal, and RNA purification. The RNA wash buffer concentrate was first reconstituted by adding 104 ml of 95% ethanol to the 24 ml container (Zymo, ZAR1054). All centrifugation steps accoutred at 10 000 x g.

HOMOGENIZATION

- To prepare the samples to be homogenised, the collected frozen tissue was thawed on ice, before adding 300 µl of lyse buffer. All samples weighed less than 20 mg, and all samples were homogenised on ice using a sonicator (Qsonica, MODEL CML-4). The sample was then cleared of lysate by centrifugation for 1 min.

CLEARING AND GDNA REMOVAL

- The supernatant was then transferred into a Spin-Away™ Filter (yellow) in a Collection Tube and centrifuged again for 1 minute for further clearing and gDNA removal. The flow through was then used for the RNA Purification steps.

RNA PURIFICATION

- Ethanol (98%) was added into the sample in a 1:1 ration to the lyse buffer, thus 300 µl was added to the sample and vortexed.
- This mixture was then transferred to a Zymo-Spin™ IIICG Column1 (green) in a Collection Tube and centrifuged for 30 seconds. This time the flow-through was discarded, but the collection tube itself was kept.
- In-column DNase I Treatment was performed to remove all trace DNA.
- The column was prewashed with 400 µl RNA Wash Buffer then centrifuged for 30 seconds and the flow-through discarded.
- 80 µl of DNase I Reaction Mix was added to each column and incubated at room temperature for 15 min. The master mix of the DNase I reaction mix was prepared in an Eppendorf tube according to the protocol shown below.
- The sample was then centrifuged for 30 seconds, 400 µl RNA Prep Buffer was added to the column and it was again centrifuged for 30 seconds. The flow-through was discarded
- 700 µl of RNA Wash Buffer was added to the column and centrifuged for 30 seconds. The flow-through was again discarded
- 400µl RNA Wash Buffer was added and centrifuged for 2 minutes to ensure complete removal of the wash buffer.
- The Zymo-Spin™ IIICG Column1 (green) columns were carefully transferred into separate 1,5 ml nuclease-free Eppendorf tubes.
- 100 µl of DNase/RNase-free water was added directly to the column matrix and centrifuged for a final 30 seconds. This concluded the RNA isolation stage. The flow through was kept on ice for cDNA synthesis preparation.

In column DNase I treatment reaction mix

DNase I Reaction Mix reagents	Volume per tube (µL)
DNase I	5
DNA Digestion Buffer	75
Total Volume	80

cDNA SYNTHESIS

Samples were converted to cDNA on the same day as the RNA isolation process using the Bio-Rad iScript™ cDNA Synthesis Kit (1708891).

- The eluted RNA concentration was measured for purity using the Nanodrop 2000 from Thermo Scientific (Roche, ZA). A purity ration ($\frac{A260}{A280}$) of 1,7 to 2,1 was considered sufficient for conversion to cDNA.
- All RNA was diluted with nuclease-free water to 50ng/µl before conversion to cDNA
- The samples were prepared in accordance to the iScript cDNA synthesis kit from BioRad in white PCR strips.
- The samples were placed into the thermocycler 2.0 (Roche, ZA) in accordance to the iScript cDNA synthesis kit from Bio-Rad.
- All the samples were then stored overnight at -20°C to be used the following day.

Reaction mix used to synthesise cDNA from the extracted RNA

Component	Volume per tube (µL)
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	5
RNA template (100fg -1µg total RNA)	10
Total Volume	20

The reaction protocol used to synthesise cDNA from the extracted RNA

Cycle	Time	Temperature
Priming	5 min	25°C
Reverse transcription	20 min	46°C
RT inactivation	1 min	95°C

AMPLIFICATION

- The PCR primers for the target (EP3R) and reference (GAPDH) genes were eluted to a 100Mm stock solution by adding nuclease-free water as per manufacturer instructions. The primer sequence and the dilution instructions can be seen below. A further 1:20 primer working dilution (50 ng/ul) was then prepared for use in the amplification. The primers were designed on Primer-BLAST and obtained from Inqaba Biotechnical Industries (Pty) Ltd.
- The reaction mix to be used for amplification was prepared in white PCR strips, in accordance to the iTaq universal SYBR Green supermix (Bio-Rad, ZA) instructions as depicted below.
- A standard cure was prepared for both the target and reference genes and run in duplicate. Samples were also run in duplicate for both primers, i.e. 2x target gene samples and 2x reference gene samples.
- A Roche LightCycler (Roche, ZA) was programmed in accordance to instruction depicted below. The reaction mixture was prepared.

PCR target and reference primers

Primer	NCBI Reference	Sequence	H_2Oµl
EP3R F	NM_012704.1	ATACCTGCTTCCCTGAGTAT	496.67
EP3R R	NM_012704.1	GAGGCCGAAAGAAGATACAA	539.66
GAPDH F	NM_017008.4	AGTGCCAGCCTCGTCTCATA	715.74
GAPDH R	NM_017008.4	GATGGTGATGGGTTTCCCGT	566.07

The PCR mix which was used

Component	Volume per tube (µL)
iTaq universal SYBR Green supermix	10
Forward primer	2
Reverse primer	2
Nuclease-free water	4
cDNA template	2
Total Volume	20

The reaction protocol used for the PCR

Cycle step	Temperature	Time (sec)	Cycles
Initial incubation	95°C	150	1
3 step Amplification	95°C	12	50
	60°C	20	
	72°C	30	
Melt curve	95°C	30	1
	65°C	60	
	97°C	1	
Cooling	40°C	10	1

ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

Quick-RNA™ Miniprep Kit

Catalog Nos. **R1054 & R1055**

Highlights

- High-quality total RNA (including small RNAs) from a wide range of samples.
- You can opt to isolate small and large RNAs in separate fractions.
- *DNA-free* RNA is ready for use in any downstream application. *DNase I included.*

Contents

ZYMO RESEARCH CORP.
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iScript™ cDNA Synthesis Kit

Catalog #	Description
1708890	**iScript cDNA Synthesis Kit**, 25 x 20 µl reactions
1708891	**iScript cDNA Synthesis Kit**, 100 x 20 µl reactions

For research purposes only.

Introduction

iScript cDNA Synthesis Kit provides a sensitive and easy-to-use solution for two-step reverse transcription quantitative PCR (RT-qPCR). This kit includes three tubes, which contain all the reagents required for successful reverse transcription.

The iScript Reverse Transcriptase is RNase H+, which provides greater sensitivity than RNase H– enzymes in qPCR. iScript is a modified Moloney murine leukemia virus (MMLV) reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided preblended with RNase inhibitor. The unique blend of oligo(dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets <1 kb in length. iScript cDNA Synthesis Kit produces excellent results in both real-time and standard RT-qPCR.

Storage and Stability

Store at –20°C. Guaranteed for 12 months at –20°C in a constant temperature freezer. Nuclease-free water can be stored at room temperature.

Note: Kits whose six-digit lot number begins with a 2 are not compatible with kits whose six-digit lot number begins with a 1. Please make note of this distinction if you have multiple lots of this kit in storage.

Kit Contents

Reagent	Volume for 25 Reactions	Volume for 100 Reactions
5x iScript Reaction Mix	100 µl	400 µl
iScript Reverse Transcriptase	25 µl	100 µl
Nuclease-free water	1.5 ml	1.5 ml

Reaction Setup

Note: The 5x iScript Reaction Mix may generate some precipitation upon thawing; this does not affect the quality of the mixture. If you do experience precipitation, please mix thoroughly to resuspend and use as directed in the following table.

Component	Volume per Reaction, µl
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	Variable
RNA template (100 fg–1 µg total RNA)*	Variable
Total volume	**20**

* When using larger amounts of input RNA (>1 µg), the reaction should be scaled up (for example, 40 µl reaction for 2 µg, or 100 µl reaction for 5 µg) to ensure optimum synthesis efficiency.

Reaction Protocol

Incubate the complete reaction mix in a thermal cycler using the following protocol:

Priming	5 min at 25°C
Reverse transcription	20 min at 46°C
RT inactivation	1 min at 95°C
Optional step	Hold at 4°C

Recommendation for Optimal Results Using the iScript cDNA Synthesis Kit

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2 µl.

Related Products

Catalog #	Description
Reverse Transcription Reagents for Real-Time qPCR	
1708840	iScript Reverse Transcription Supermix for RT-qPCR
1725037	iScript Advanced cDNA Synthesis Kit for RT-qPCR
1708896	iScript Select cDNA Synthesis Kit
1725034	iScript gDNA Clear cDNA Synthesis Kit
Reagents for Real-Time qPCR	
1725270	SsoAdvanced™ Universal SYBR® Green Supermix
1725280	SsoAdvanced Universal Probes Supermix
1725120	iTaq™ Universal SYBR® Green Supermix
1725130	iTaq Universal Probes Supermix
1725160	SsoAdvanced PreAmp Supermix

Visit **bio-rad.com/web/iscriptcDNA** for more information.

BIO-RAD

iTaq™ Universal SYBR® Green Supermix

Catalog #	Description
1725120	**iTaq™ Universal SYBR® Green Supermix**, 2 ml (2 x 1 ml vials), 200 x 20 µl reactions
1725121	**iTaq™ Universal SYBR® Green Supermix**, 5 ml (5 x 1 ml vials), 500 x 20 µl reactions
1725122	**iTaq™ Universal SYBR® Green Supermix**, 10 ml (10 x 1 ml vials), 1,000 x 20 µl reactions
1725124	**iTaq™ Universal SYBR® Green Supermix**, 25 ml (5 x 5 ml vials), 2,500 x 20 µl reactions
1725125	**iTaq™ Universal SYBR® Green Supermix**, 50 ml (10 x 5 ml vials), 5,000 x 20 µl reactions

For research purposes only.

Storage and Stability

Guaranteed for 12 months in a constant temperature freezer at –20°C protected from light. For convenience, this supermix can be stored at 4°C for up to 3 months.

Kit Contents

iTaq™ Universal SYBR® Green Supermix is a 2x concentrated, ready-to-use reaction master mix optimized for dye-based quantitative PCR (qPCR) on any real-time PCR instrument (ROX-independent and ROX-dependent). It contains antibody-mediated hot-start iTaq DNA Polymerase, dNTPs, $MgCl_2$, SYBR® Green I Dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein).

Instrument Compatibility

This supermix is compatible with all Bio-Rad and other commercially available real-time PCR systems.

Reaction Mix Preparation and Thermal Cycling Protocol

1. Thaw iTaq™ Universal SYBR® Green Supermix and other frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solutions at the bottom of tubes, then store on ice protected from light.
2. Prepare (on ice or at room temperature) enough reaction mix for all qPCR reactions by adding all required components, except the DNA template, according to the recommendations in Table 1.
3. Mix the reaction mix thoroughly to ensure homogeneity and dispense equal aliquots into each qPCR tube or into the wells of a qPCR plate. Good pipetting practice must be employed to ensure assay precision and accuracy.
4. Add DNA samples (and nuclease-free H_2O if needed) to the PCR tubes or wells containing reaction mix (Table 1), seal tubes or wells with flat caps or optically transparent film, and vortex 30 sec or more to ensure thorough mixing of the reaction components. Spin the tubes or plate to remove any air bubbles and collect the reaction mixture in the vessel bottom.
5. Program the thermal cycling protocol on a real-time PCR instrument according to Table 2.
6. Load the PCR tubes or plate into the real-time PCR instrument and start the PCR run.
7. Perform data analysis according to the instrument-specific instructions.

Table 1. Reaction setup.*

Component	Volume/20 µl Reaction, µl	Volume/10 µl Reaction, µl	Final Concentration
iTaq™ Universal SYBR® Green Supermix (2x)	10	5	1x
Forward and reverse primers	Variable	Variable	300–500 nM each primer
DNA template (add at step 4)	Variable	Variable	cDNA: 100 ng–100 fg Genomic DNA: 50 ng–5 pg
Nuclease-free H_2O	To 20 µl	To 10 µl	—
Total reaction mix volume	**20 µl**	**10 µl**	**—**

* Scale all components proportionally according to sample number and reaction volumes.

Table 2. Thermal cycling protocol.

Real-Time PCR System	Setting/ Block	Polymerase Activation and DNA Denaturation at 95°C	Amplification			Melt Curve Analysis
			Denaturation at 95°C, sec	Annealing/Extension and Plate Read at 60°C, sec	Cycles	
Bio-Rad® CFX96™, CFX384™, CFX96 Touch™, CFX96 Touch Deep Well, CFX384 Touch™, CFX Connect™	SYBR® only	20–30 sec for cDNA or 2–5 min for genomic DNA	2–5	15–30	35–40	65–95°C 0.5°C increments at 2–5 sec/step (or use instrument default setting)
Bio-Rad® iQ™5, MiniOpticon™, Chromo4™, MyiQ™	Standard		10–15	15–30		
Applied Biosystems 7500, 7900HT, QuantStudio, StepOne, StepOnePlus, and ViiA 7	Fast		1–3	20–30		
	Standard		15	60		
Applied Biosystems 7300 and 7000	Standard		15	60		
Roche LightCycler 480	Fast		2–5	15–30		
	Standard		15	60		
QIAGEN Rotor-Gene and Stratagene Mx series	Fast		2–5	15–30		

Recommendations for Primer Design

- The iTaq™ Universal SYBR® Green Supermix and the qPCR cycling protocols have been optimized for assays with a primer melting temperature (T_m) of 60°C and designed using the open source Primer3 program (**http://frodo.wi.mit.edu/**) under its default settings. For assays designed using other tools, the primer T_m should be recalculated using Primer3 for determining annealing/extension temperature
- For best qPCR efficiency, design assays targeting an amplicon size of 70–150 bp. For amplicons >250 bp in length or with high GC or AT content, longer annealing/ extension times (30–60 sec) can be used

Quality Control

iTaq™ Universal SYBR® Green Supermix demonstrates high PCR efficiency and a wide linear dynamic range. Stringent specifications are maintained to ensure lot-to-lot consistency. This product is free of detectable DNase and RNase activities.

Related Products

Reverse transcription reagents for real-time qPCR:

- iScript™ Reverse Transcription Supermix for RT-qPCR (1708840)
- iScript Advanced cDNA Synthesis Kit for RT-qPCR (1725037)
- iScript gDNA Clear cDNA Synthesis Kit (1725034)
- iScript cDNA Synthesis Kit (1708890)

Reagents for real-time qPCR:

- SsoAdvanced™ Universal SYBR® Green Supermix (1725270)
- iTaq™ Universal SYBR® Green One-Step Kit (1725150)

PCR primer and probe assays for real-time qPCR:

- PrimePCR™ Assays and Panels

Visit **bio-rad.com/amplification** to learn more about Bio-Rad's complete solution for amplification.

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APPENDIX F

A few personal photos taken throughout the study.

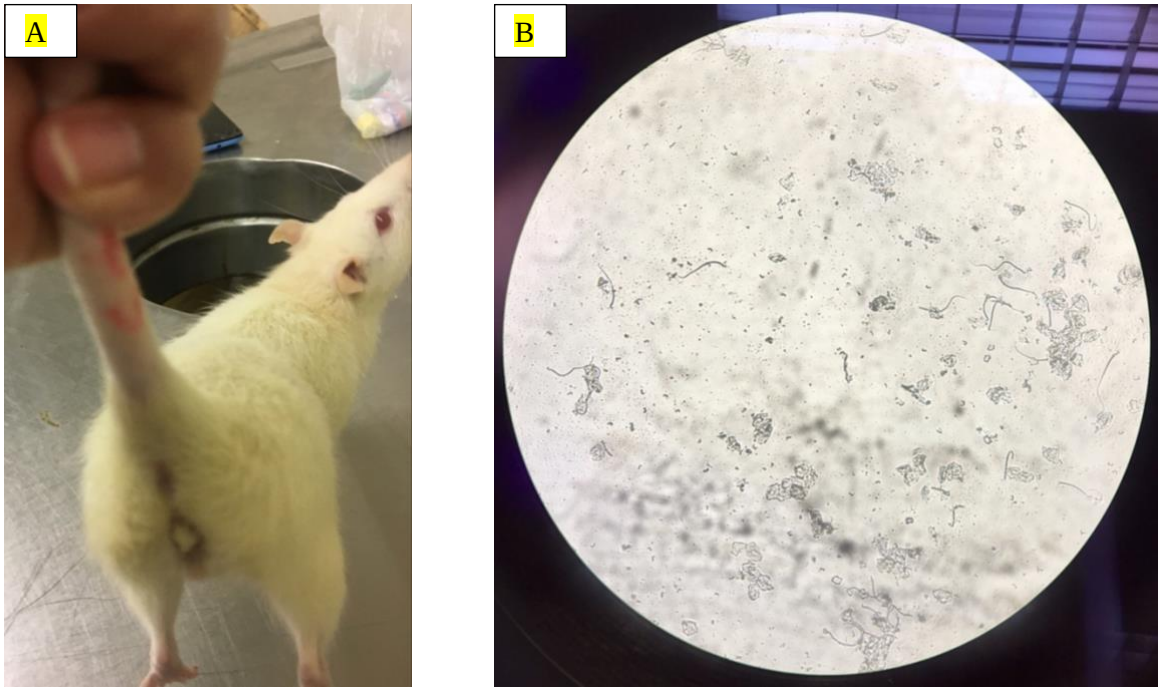

Figure 10.1. Photo A on the left is of a vaginal plug in the vaginal orifice of a female Sprague Dawley rat the morning after mating. Photo B on the right is a vaginal smear preformed after successful mating, sperm and nucleated epithelial cells are visible.

Figure 10.2. The water bath used to create the hyperthermic environment (H) model. A single rat pup was placed into the plastic container lowered into the hot water. The lid and the upper rim of the container were aerated. The bottom of the container was lined with polystyrene to prevents the pup's paws from burning.

Figure 10.3. A group photo of the UKZN Neuroscience and ZuluCortex Club members promoting brain awareness in local school communities.

Figure 10.4. A photo of my bored fiancé pretending to be Thanos with the infinity stones, while keeping me company at 2 am during my PCR runs.