Prenatal Stress & Febrile Seizures Effects on the Epigenetic Mechanisms Involved in Cognitive Function Following Treatment with *Searsia Chirindensis*

BY

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Preface

The experimental studies carried out in this thesis were conducted in the Neuroscience Labs - and the Biomedical Resource Centre of the School of Laboratory Medicine and Medical Sciences at the University of Kwa-Zulu Natal (Westville Campus), under the supervision of Dr. M. V. Mabandla and Ms L. Qulu.

Declaration

I, Sadiyah Cassim (student number: 213541708), hereby declare that the dissertation entitled **Prenatal Stress & Febrile Seizures Effects on the Epigenetic Mechanisms Involved in Cognitive Function Following Treatment with *Searsia Chirindensis*** is the result of my own investigation and research and that it has not been submitted in part or full for any other degree or to any other University or Tertiary Institution. Where use was made of others work, it has been duly acknowledged. The research done in this study was carried out under the supervision of Dr. M. V. Mabandla and Ms. L. Qulu.

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Abbreviations

α - alpha

β - beta

κ - kappa

γ - gamma

μ - micro

HSD-2 - hydroxysteriod-dehydrogenase 2

AChE - acetylcholine esterase

ACh - acetylcholine

ACTH - adrenocorticotropic hormone

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF - brain-derived neurotrophic factor

BGT-1 - betaine-GABA transporter 1

CA - cornus ammonis

CNS - central nervous system

CD – cluster of differentiation

COX-2 - cyclooxygenase-2

CPG - cytosine-phosphate-guanine

CRH - corticotrophin-releasing hormone

DNA - deoxynucleic acid

DNMTs - DNA methyltransferases

EAATs - excitatory amino acid transporters

EPSPs – excitatory post-synaptic potentials

FS - febrile seizure

GABA - γ-aminobutyric acid

GAD - glutamic acid decarboxylase

GC - glucocorticoids

GND - gestational day

GR - glucocoticoid receptors

HATs - histone acetyltransferases

HDACs - histone deacetylases

HPA - hypothalamic-pituitary-adrenal

i.p. - intraperitoneally

IPSP - inhibitory post-synaptic potential

IL- interleukin

IL-r1a - interleukin-receptor 1 antagonist

KAR - kainate receptor

Kg - kilogram

LPS - lipopolysaccharides

LTD - long term depression

LTP - long term potentiation

mAChRs - muscarinic acetylcholine receptors

MAPK - mitogen-activated protein kinases

MeCP2 - methyl-CPG-binding protein 2

mg - milligram

MR - mineralocorticoid receptors

nAChRs - nicotinic acetylcholine receptors

NF - nuclear factor

NMDA - N-methyl-D-aspartate

NS - non-stressed

PBS - phosphate buffered saline

PFC - prefrontal cortex

PGs - prostaglandins

PND - postnatal day

proBDNF - precursor protein brain-derived-neurotrophic factor

REST - repressor-element-1-silencing-transcriptional factor

RNA – ribonucleic acid

S - stress

SNARE - SNAP (soluble NSF attachment protein) Receptor

TGF - transforming growth factor

TLR - toll-like-receptor

TNF - tumour necrosis factor

Trk - tropomyosin-related kinase

UBE3 - ubiquitin-protein ligase 3

VGlut3 - vesicular glutamate transporter 3

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Study Outline

This dissertation is presented in article format; with three studies and six chapters. A background has been included in order to orientate the reader to the overall view of the problem statement as well as our aims. Chapter one is a literature review which is a general discussion on key concepts investigated in the study. Chapter two contains work published in "Neurobiology of Learning and Memory". Sadiyah Cassim, Lihle Qulu & Musa V. Mabandla. Neurobiology of Learning and Memory (2015) 125:195-201. DOI:10.1016/j.nlm.2015.09.002. The published version of the article has been attached as addendum A. Chapter three entails study two of the project. This chapter is currently under review in the journal "Annals of Neuroscience". Sadiyah Cassim, Lihle Qulu & Musa V. Mabandla. The submitted copy of the article has been attached as addendum B. Chapter four is the third study of the project, this study is prepared in the article format required for the journal "Phytomedicine". Chapter five is a combined conclusion for all three studies conducted in this dissertation. Chapter six consists of the reference list for all the work submitted.

Abstract

Febrile seizures are of growing concern in the health fraternity within the African continent. These seizures predominantly affect children between the ages of 3 months and 5 years of age. Stress during pregnancy may influence the functioning of the hypothalamic-pituitary-adrenal (HPA) axis in the developing foetus. This has a tendency to increase the brain's vulnerability to a number of insults including febrile seizures. Current treatment for febrile seizures often has undesirable side effects hence the need to find more effective alternatives. *Searsia chirindensis* (*Searsia*) is commonly used to treat conditions associated with the heart, rheumatism and brain abnormalities. The focus of this study was to investigate whether exposure to prenatal stress and early life febrile seizures may affect the expression of genes that play a role in neuronal plasticity. For this we looked at the expression of the MeCP2 and REST genes in the hippocampus. Furthermore, we investigated whether neuronal malformations and the resulting cognitive deficits associated with exposure to early life stress influences the concentration of the neurotrophic factor, BDNF and whether further exposure to febrile seizures exacerbates the stress effect. We also investigated whether treating febrile convulsions with *Searsia* attenuates the negative effects caused by stress on febrile seizure development as well as any cognitive effects that may be caused by the exposure to stress and febrile seizures. Fourteen day old (PND 14) pups were divided into the following groups 1) Normally reared Sprague-Dawley offspring injected with saline (NSS). (2) Prenatally stressed offspring injected with saline (SS). (3) Normally reared offspring with febrile seizures (NSFS). (4) Prenatally stressed offspring with febrile seizures (SFS). (5) Normally reared offspring treated with *Searsia* (NS-S). (6) Prenatally stressed offspring treated with *Searsia* (S-S). (7) Normally reared offspring with febrile seizures and treated with *Searsia* (NSFS-S). (8) Prenatally stressed offspring with febrile seizures and treated with *Searsia* (SFS-S). Lipopolysaccharide and kainic acid were used to induce the febrile seizures. The Morris water maze (MWM) was used to assess learning and memory function and the elevated plus maze (EPM) was used to assess anxiety-like behaviour in these young rats. MeCP2/REST genes expression and the concentration of BDNF as well as AChE were quantified using an immunoassay on hippocampal tissue. Our results showed that exposure to prenatal stress (SS) and febrile seizures (NSFS) may impair cognitive behavioural function in the short-term. However, in the NSFS animals, there seems to be an attempt to counteract the effects of febrile seizures with time. Furthermore, exposure to prenatal stress impeded the release of the neurotrophic factor, BDNF, while attenuating REST gene expression in febrile seizure animals. These factors may contribute to the hindering of neurogenic properties in the young, thus leading to neuronal plasticity deficits and possibly cognitive malfunction in later life. Treatment with *Searsia* attenuated the transient cognitive impairments present following a seizure by influencing the concentration of hippocampal acetylcholine and activating MeCP2 gene expression. This suggests a role for *Searsia* in the management of febrile seizures and in attenuating the development of chronic cognitive deficits.

Background

Febrile seizures arise from a systemic fever occurring in the body due to an infection which evokes the excessive firing of neurons (Qulu, *et al.*, 2012; Marchi, *et al.*, 2013). Febrile seizure are triggered by infections such as *otitis media*, *otitis externa*, *sinusitis*, *pharyngitis* and *conjunctivitis* (Alter, *et al.*, 2011). These infections trigger the activation of the immune system thus promoting the release of various cytokines and leucocytes (Marchi, *et al.*, 2013). This subsequently results in neurotransmitter (glutamate and γ-aminobutyric acid (GABA) imbalance provoking a trigger of a seizure (Batten, 2013; Lason, *et al.,* 2013).

Furthermore, exposure to early life stress has been shown to result in the malformation of neuronal development in young rats (McEwen, 2012). Prenatal stress is a critical component of early life stressors which is a result of stress experienced by pregnant mothers in their last trimester of pregnancy (Moisiadis & Matthews, 2014). This form of stress has been shown to exacerbate cytokine production and seizure progression in rat offspring (Qulu, *et al*., 2012) resulting in hippocampal mass reduction (Qulu, *et al*., 2015), increase in excitatory amino acids (glutamate and acetylcholine) that subsequently affects neuronal function (McEwen, *et al.*, 2015). The implications of neuronal loss particularly in the hippocampus has been show to affect neuronal networks from histological to molecular aspects in brain structure thereby affecting brain function (McEwen, 2012).

Furthermore, studies have shown that various genomic alterations may be a link to abnormal behaviour and functioning in developing brains (Qureshi & Mehler, 2010; Lubin, 2012). Febrile seizures have been shown to play an important role in influencing transcriptional factors and gene expressions *viz.* $GABA_A$ receptor unit, REST, CREB, MeCP2 and BDNF, that contribute to changes in neuronal networks particularly in the hippocampus (Qureshi & Mehler, 2010; Lubin, 2012; Roopra, *et al.*, 2012).

Treatment regimens undertaken to treat various febrile seizures include sodium valproate, carbamazepine and phenobarbitone (Lui, *et al.*, 2015). However these treatments have adverse side effects and financial implications, many have resorted to the use of traditional medicinal plants as a source of alternative treatment (Lui, *et al.*, 2015). *Searsia chirindensis* is amongst the few plants used to treat various disorders inclusive of convulsions (Ojewole, 2008).

Therefore the focus of our study was to investigate:

1. The role of the MeCP2 and REST genes in the developing brain by investigating the effects of early life exposure to neuronal insults (prenatal stress and febrile seizures) on learning and memory as well as determining the role of MeCP2/REST genes activation on hippocampal function.
2. Whether neuronal malformations and the resulting cognitive deficits associated with stress are linked to changes in BDNF concentration and the REST gene factor expression. We

further investigated whether exposure to febrile seizures exacerbates the changes seen in these factors, and

3. We investigated the effects of *Searsia* crude bark methanolic extract on prenatally stressed and febrile seizure exposed rats that exhibit cognitive impairment and whether the crude bark methanolic extract attenuates the changes observed in the hippocampus.

Chapter One

Literature Review

1. Epidemiology

Seizures are clinical symptoms that advocate a diagnosis for a condition that may be related to epilepsy or clinical manifestation related to non-epileptic conditions such as febrile seizures (Marchi, *et al.*, 2014). Febrile seizures arise from a systemic fever occurring in the body due to an underlying infection which evokes the excessive firing of impulses (Marchi, *et al.*, 2014). Febrile seizures can be classified into three categories *viz.* (1) simple febrile seizures, (2) complex febrile seizures and (3) status epilepticus (Cappovilla, *et al.*, 2009; Heida, *et al.*, 2009). Simple febrile seizures occur predominantly in children of 3 months to 5 years of age, with a seizure duration lasting between 1-2 minutes but not more than 15 minutes and a clear focus of an infection is present (Cappovilla *et al*, 2009). Complex febrile seizures are usually prolonged seizures that are focalised and last greater than 15 minutes with chances of recurring within 24 hours (Cappovilla *et al*, 2009). Status epilepticus lasts greater than 30 minutes, with having two unprovoked seizures occurring 24 hours apart (Fisher, *et al.*, 2014).

Infants and toddlers between the age of 3 months and 5 years old are more susceptible to a febrile seizure (Cappovilla *et al*, 2009; Ackermann & Van Tooran, 2012). Factors such as poor sanitation and inadequate medical facilities, as well as malnutrition are common issues affecting developing countries (Idro, *et al,* 2008). Developing countries include predominantly parts of East Africa (Mozambique, Ethiopia, Kenya and Sudan), followed by parts of West Africa (Nigeria, Democratic Republic of Congo and Mali) and last, Southern Africa (Zimbabwe, Botswana and South Africa) (Figure 1). These situations allow for easy contraction of infections, namely- gastroenteritis and respiratory tract infections which subsequently activates the body's immune responses resulting in systemic inflammation (Heida, *et al.*, 2009; Ackermann & Van Tooran, 2012).

South Africa has been shown to be affected by febrile seizures with Northern Province having a high prevalence of approximately 6.7/1000 reported cases (Ackermann & Van Tooran, 2012). Thus it is confirmed that febrile seizures are a growing concern in many facets of the health fraternity within South Africa.

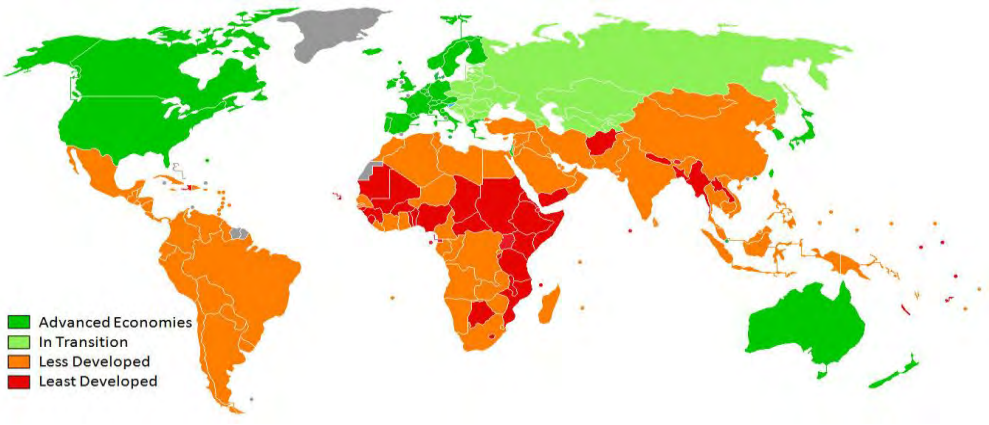

Figure 1. Geographical illustration depicting the various developing countries (Adapted from Manarrazi Media Press, 2014).

2. Febrile Seizures

In the 5th century BC, Aulus Cornelius Celsus, a Roman writer and physician, was the first person to describe inflammation. He defined inflammation as a localized protective response caused by an injury or destruction of tissue that serves to destroy or wall off both the infectious agent (bacteria, virus, parasite or spores) and the tissue it affects (Scott, 2004; shah, 2012; Evans, *el al.*, 2015). The inflammatory response is evoked by physical, chemical or biological/infectious agents that result in symptoms of heat (sometimes causing a fever), redness, swelling and pain (Scott, 2004; Stankov, 2012;Vezzani, *et al.*, 2013; Evans, *el al.*, 2015). The immune system is the bodies line of defence that protects it from foreign invaders by triggering an inflammatory response that can be classified into innate immunity (natural resistance/immunity the body has against pathogens) and adaptive immunity (acquired immunity the body has to acquire to resist pathogens) (Hoeba, *et al.*, 2004).

Innate immunity involves a non-specific reaction of the body to a particular pathogen (Hoeba, *et al.,* 2004). This natural immunity of the body is dependent on a group of proteins (cytokines) and phagocytic cells (macrophages, neutrophils and dendritic cells) to recognize foreign invaders with the subsequent activation to destroy them upon entry (Hoeba, *et al.,* 2004). However, this first line of defence does not produce memory cells to allow the body to become immune to a pathogen hence the interplay of the adaptive immunity is required (Hoeba, *et al.,* 2004). The adaptive immunity creates "immunological memory" of a response to pathogen that enhances the body to defend itself in the long run (Hoeba, *et al.,* 2004).

Cytokines are signalling proteins that are grouped according to acute or chronic inflammation (Feghali & Wright, 1997; Galic, *et al.* 2012). Acute inflammation is a hallmark of the immediate response of the body to an infection by releasing cytokines; Interleukin (IL)-1β, Tumor Necrosis Factor-α (TNF-α), IL-6, IL-8 and various other stimulating factors (Feghali & Wright, 1997). While chronic inflammation include IL-3, IL-4, IL-5, IL-7, IL-9, IL-10, IL-13 and various other immune factors (Feghali & Wright, 1997). The cytokines are further divided into pro-inflammatory and anti-inflammatory factors (Feghali

& Wright, 1997; Galic, *et al.* 2012). Both IL-1β, IL-6 and TNF-α are cytokine factors produced at the site of inflammation and their net effect is often taken into consideration by making a correlation between the levels of expression and disease severity (Dinarello, 2000).

During a febrile seizure, an infection activates the immune system leading to an inflammatory response with the subsequent release of immune cells such as macrophages and neutrophils (Dubé *et al.,* 2009). This subsequently results in the release of cytokines such as IL-6, TNF-α and predominantly, IL-1β (figure 2) (Dubé *et al.*, 2009). IL-1β is a pro-inflammatory cytokine that plays a pivotal role in the progression of febrile seizures in response to the underlying infection (Heida *et al.,* 2009). High levels of circulating peripheral cytokines compromise the endothelial cells of the blood brain barrier resulting in a leaky blood brain barrier (Heida *et al.,* 2009). Levels of IL-1β are high and therefore its binding is favoured and this subsequently sets off a sequelae of events that lead to the stimulation of the enzyme cyclooxygenase 2 (COX-2) to catalyse the conversion of arachidonic acid into prostaglandin E_2 (PGE_2) (figure 2) (Dubé *et al.,* 2009; Heida *et al.,* 2009). This results in the raise of the body's thermostat "set point" in the hypothalamus resulting in a rise in core body temperature (fever) (Heida *et al.,* 2009). IL-1β has a naturally occurring antagonist, the anti-inflammatory cytokine, IL-1 receptor antagonist (IL-1Ra) which modulates the pro-inflammatory effects of IL-1β by binding to its receptors and inhibiting the effects in normal function to bring about a homeostatic balance between pro- and anti-inflammatory cytokines (Heida *et al.,* 2009). However, during a febrile seizure both IL-1β and IL-R1 compete for the same binding site favouring IL-1β resulting in a hyper secretion of IL-1β (Heida *et al.,* 2009). Furthermore, IL-1β phosphorylates glutamate receptors leading to an increase in cell permeability to calcium ions and subsequently decreasing $GABA_A$-mediated currents (Heida *et al.,* 2009). This results in excessive neuronal excitability which involves the imbalance of the neurotransmitters, glutamate which is excitatory, and GABA which is inhibitory (figure 2) (Dubé *et al.,* 2005, Heida *et al.,* 2009). In addition, excessive levels of IL-1β has also been shown to be neurotoxic particularly to the hippocampus which subsequently affects cognitive function (Vezzani, *et al.,* 2011; Marin & Kipnis, 2013).

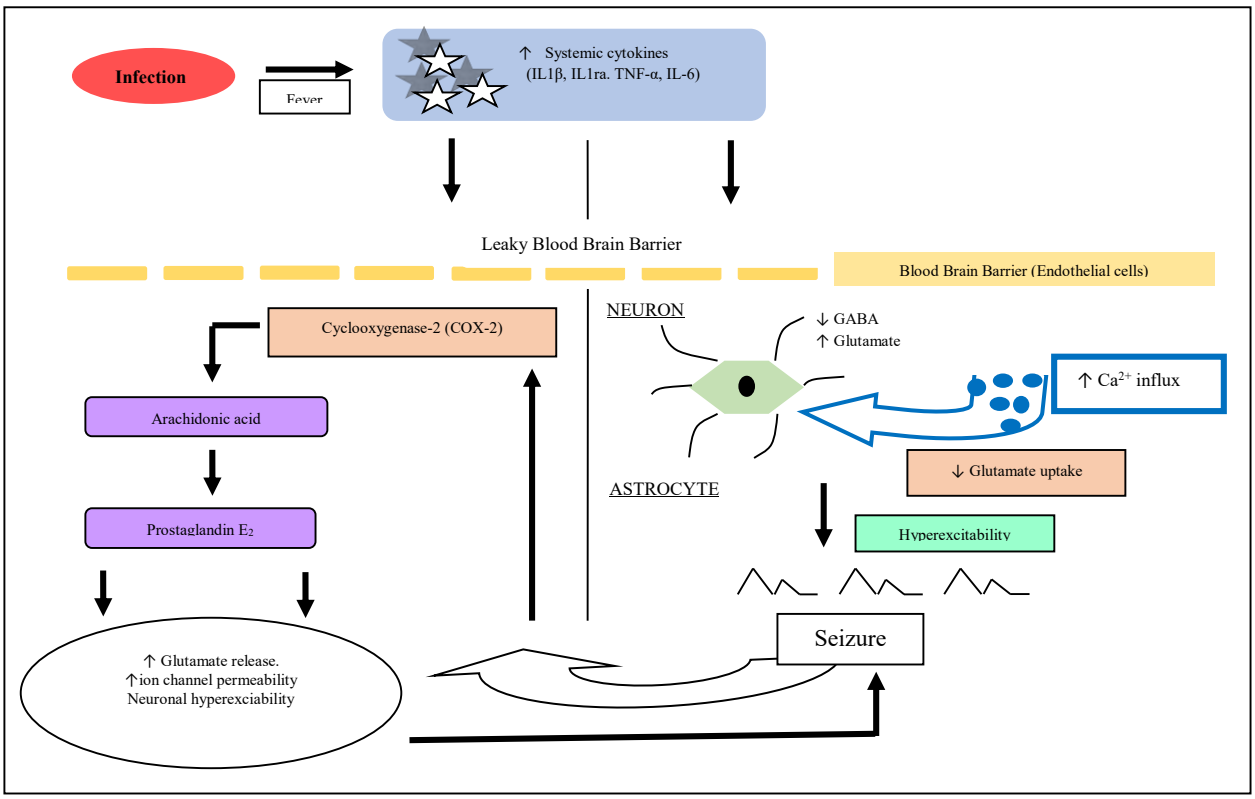

Figure 2. Projected pathways of neuroinflammation. An infection causing a fever, triggers a systemic increase in inflammatory markers thereby resulting in a leaky BBB. This results in part A (left), activating the COX-2 pathway subsequently leading to a seizure and part B (right), directly affecting neuronal function, causing a seizure which subsequently promotes the COX-2 pathway.

Glutamate is an excitatory amino acid that plays a pivotal role in neuronal differentiation and migration in developing circuitries of the brain (Danbolt, 2001, Ben-Ari & Holmes, 2006). It is particular important for brain function that involves cognition, memory and learning (Danbolt, 2001). It is also responsible for the direct signal transmission between neurons whereby it acts on ionotropic and metabotropic glutamate receptors respectively (Danbolt, 2001, Ben-Ari & Holmes, 2006). Ionotropic glutamate receptors are dependent on ionic gradients whereas metabotropic receptors facilitate their action coupled to secondary G-protein messengers (Danbolt, 2001). The ionotropic receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate (KARs) receptors (Popoli, *et al.*, 2012). Glutamatergic transmission predominantly occurs within the tripartite synapse of glutamate presenting cells (Popoli, *et al.*, 2012). Glutamate is present in extremely high levels to ensure that tight regulatory processes are in place to allow modulation of glutamate receptors to optimally transmit impulses without causing excitotoxicity (Popoli, *et al.*, 2012).

In relation to febrile seizures, the subsequent effect of exacerbated levels of IL-1β affects the NMDA receptors resulting in the influx of calcium ions (Popoli, *et al.*, 2012; Lason, *et al.,* 2013). This effect causes an increase in neuronal firing that triggers a seizure (Popoli, *et al.*, 2012; Lason, *et al.,* 2013).

Subsequently resulting in excitotoxicity within neuronal cells thereby affecting neuronal network functioning with detrimental effects on learning and memory in particular (Popoli, *et al.*, 2012; Lason, *et al.,* 2013).

The hippocampus is involved in the learning and memory process with the *cornus ammonis*-1 region and dentate gyrus expressing an abundant of AMPA and NMDA receptors, respectively (Riedel, *et al.,* 2003; Lason, *et al.,* 2013). Long-term synaptic potentials are a possible mechanism involved for learning and memory in which AMPA receptor-mediated depolarization "knocks off" magnesium ions that block the NMDA receptors at resting membrane potential allowing for the influx of calcium ions (Lason, *et al.*, 2013). Depending on the calcium concentration intracellularly, this subsequently activates protein kinases or phosphatases that lead to activation of various downstream signalling pathways (Lason, *et al.*, 2013). AMPA receptors generates fast excitatory post-synaptic potentials, allowing for the permeability of sodium, potassium and limited calcium ions (Lason, *et al.*, 2013). NMDA receptors are responsible for the phosphorylation of AMPA receptors, which increases ion flow thereby resulting in an increase in synaptic contacts via new dendritic out-growths (Riedel, *et al.,* 2003; Popoli, *et al.*, 2012; Lason, *et al.,* 2013). This subsequently contributes to learning and the consolidation of short-term memory to long-term memory (Riedel, *et al.,* 2003). In addition to excitatory amino acid, glutamate, there is also an inhibitory amino acid that balances neuronal firing.

GABA is the main inhibitory amino acid of the central nervous system which acts predominantly on chloride channels, $GABA_A$ ionotropic receptor, to initiate an inhibitory postsynaptic potential (IPSP) (Lason, *et al.*, 2013). This initiation acts as a negative feedback mechanism in prevention of excessive excitatory discharges stimulated by glutamate in neurons particularly in events of a seizure (Lason, *et al.*, 2013). In contrast, impairment to the GABAergic system may compromise neural network function thus contributing to seizure susceptibility (Lason, *et al.*, 2013). Therefore, it is critical to maintain a balance of GABA in the brain as Ben-Ari has postulated that increased glutamate and decreased GABA in principal cells within the hippocampus are accompanied by persistent increase of intracellular levels of chloride ions contributing to febrile seizure development. Thus it has been well established that $GABA_A$ receptors are present intra- and extra-synaptically throughout the brain which are likely to control seizure thresholds (Lason, *et al.*, 2013). Furthermore, acetylcholine (ACh) has been shown to influence the release GABA and glutamate respectively (Granger, *et al.*, 2016).

2.1. The role of Acetylcholine (ACh) in neurotransmission

ACh is considered as an excitatory neurotransmitter that is responsible for the facilitation of learning and memory formation and it increases alertness and attention (Granger, *et al.*, 2016). The hippocampus, an area of the limbic system within the brain, is highly innervated with extrinsic and intrinsic inputs from cholinergic neurons (Granger, *et al.*, 2016). This area is also rich with ionotropic glutamate receptors and therefore it has been shown that ACh activates these receptors in addition to

nictonic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) (Granger, *et al.*, 2016). Activation nAChRs results in the increased release of glutamate and GABA (Radcliffe, *et al.*, 1999). This is due to nAChRs having a high permeability to calcium ions relative to the NMDA receptors (Radcliffe, *et al.*, 1999). ACh receptors regulate post-synaptic cellular excitability and synaptic release of neurotransmitters at the presynaptic cell thereby directly altering synaptic plasticity (Granger, *et al.*, 2016). Glutamate receptor, VGlut3, is a co-transmitter with ACh particularly in the striatum (Granger, *et al.*, 2016). In the hippocampus, serotonergic neurons innervate the striatum VGlut3 transporters thereby promoting the co-transmission of glutamate and ACh release making the hippocampus the area of the brain susceptible to neuronal insults such as febrile seizures (Granger, *et al.*, 2016).

It has been further demonstrated that high levels of glucocorticoids influence neurotransmitter release mainly in the prefrontal cortex (PFC) and the hippocampus (Popoli, *et al.*, 2011). This suggests that various forms of stress also influences neuronal function with subsequent malformation in plasticity and functionality.

3. The effects of stress on brain function

Stress is a term often used to describe the ability of the human body and function to adapt in presence of adverse life changing events *viz.* abuse, trauma and various underlying environmental factors (McEwen, 2008). There are different types of stressors particularly in the evolving age of poverty, malnutrition, famine and war, many children are subjected to loss of maternal care (maternal separation stress), or the inability to cope with situations at a young age and sometimes having been exposed to stress in utero (prenatal stress) (McEwen, 2008).

3.1. Prenatal Stress

Prenatal stress is a term used to describe the stress response experienced on the developing foetus by an expectant mother (Lemaire, *et al.*, 2000).

Stress is regulated via the hypothalamic-pituitary-adrenal (HPA) axis and is responsible for the production and secretion of corticosteroids under basal and stressed conditions (Moisiadis & Matthews, 2014a). The paraventricular nucleus within the hypothalamus synthesizes corticotrophin-releasing hormone (CRH) which stimulate adrenocorticotropin hormone (ACTH) from the anterior pituitary into the blood circulation (Moisiadis & Matthews, 2014a). This results in the release of glucocorticoids (cortisol in humans and corticosterone in rats) that bind specifically to glucocorticoid and mineralocorticoid receptors in the hippocampus, the paraventricular nucleus and the anterior pituitary (Moisiadis & Matthews, 2014a). Cortisol feedback is responsible for the feed-forward mechanism which promotes the HPA-axis to activate the glucocorticoid and mineralocorticoid receptors within the hippocampus, the paraventricular nucleus and the anterior pituitary when glucocorticoid levels drop

(Moisiadis & Matthews, 2014a). However, increase in glucocorticoid levels leads to a retardation in the HPA activity (Moisiadis & Matthews, 2014a).

3.1.1. Effects of prenatal stress on the developing foetus

During pregnancy circulating maternal glucocorticoids cannot easily cross the placental membrane (Barbazanges, *et al*., 1996; Seckl, 2004). Under normal physiological conditions, foetal glucocorticoid concentrations are high during the last trimester of the gestation period, as it is required for the development of organ systems in the foetus (Challis, *et al.*, 2001). However, exposure to prenatal stress exacerbates levels of foetal glucocorticoids by impairing the negative feedback mechanism involving 11β- hydroxysteriod-dehydrogenase type 2 (11β-HSD2) (Challis, *et al.*, 2001). 11β-HSD2 is an enzyme responsible for preventing the glucocorticoid effects on the developing HPA-axis of the foetus under prolonged exposure to glucocorticoids (Seckl, 2004; Harris & Seckl, 2010; de Kloet, *et al.*, 2014). 11β-HSD2 catalyses the inactivation of glucocorticoids (cortisol and corticosterone) to inert 11-keto forms (cortisone and 11-dehdrocorticosterone) (Moisiadis & Matthews, 2014a). Therefore the presence of this enzyme acts as a shielding mechanism from higher than normal levels of exposure to glucocorticoids present in the maternal circulation (Meaney, *et al.*, 2007). Since this enzyme is least active during the last trimester of pregnancy and cortisol in humans and corticosterone in rats, is required for lung maturation and parturition, exposure to exacerbated levels of cortisol/corticosterone saturates the binding to glucocorticoid receptors resulting in excess circulating glucocorticoids subsequently leading to compromised neuronal development and function within the brain of the foetus (Welberg & Seckl, 2001; Harris & Seckl, 2010; de Kloet, *et al.*, 2014).

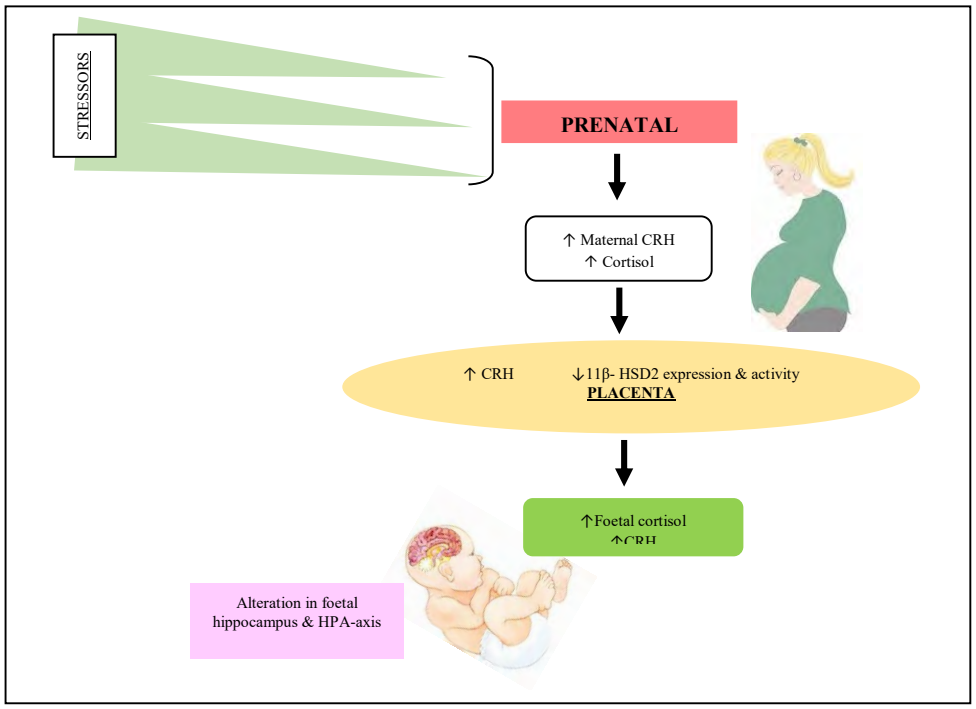

Figure 4. Mechanism and effect of prenatal stress on developing circuitries in a new-born.

Studies have shown that prolonged exposure to glucocorticoids has deleterious effects on various areas of the brain especially in the developing foetus (McEwen & Sapolsky, 1995; Sapolsky, 2003; Champagne, *et al.*, 2009; Harris, *et al.*, 2011). Glucocorticoids exerts its effects by binding to glucocorticoid receptors (promotes negative feedback mechanism) and mineralocorticoid receptors (promotes positive feedback mechanism) in the brain, particularly the hippocampus where these receptors are in abundance (McEwen, 2008; Harris, *et al.*, 2011). Binding influences many factors in the developing brain specifically gene expression and transcription (Harris, *et al.*, 2011). It is also responsible for the promotion of correct brain development by initiating terminal maturation, remodelling of axons and dendrites and overall neurogenesis (West & Greenberg, 2011). Mothers who self-reported severe stress during pregnancy gave birth to offspring showing temperamental and behavioural problems, as well as impaired cognition at the age of 7 years old (Gutteling, *et al.*, 2005; Gutteling, *et al.*, 2006). Animal studies have also reported that prenatal stress (rodent restraint stress) affected birth weight, cognitive function, anxiety-levels and altered functional brain development (McEwen & Sapolsky, 1995; McEwen, *et al.*, 2001; Qulu, *et al.*, 2015a). There has also been reports of abnormal physiologic function due to excessive exposure to glucocorticoids *viz.* immune incompetence, inflammatory responses, bone and intermediary metabolic syndromes and cardiovascular malfunction (Champagne, *et al.*, 2009). Therefore, prenatal stress can be seen to be detrimental specifically to the limbic circuitry of the brain.

Signals from the limbic circuitry reach afferent neurons in the hypothalamus, which promotes the release of corticotrophin releasing hormone (CRH) and peptidergics namely vasopressin, prolactin and growth hormone, which in return exerts its effects on behavioural, autonomous and neuroendocrine responses to the stressor (Champagne, *et al.*, 2009). Cortisol in humans and corticosterone in rodents, is the end products of the HPA-axis and are responsible for promoting synaptic plasticity and adaptivity to stressful events; allostasis (McEwen & Wingfield, 2003). Furthermore, glucocorticoid exposure have also been shown to have an influence on neurotransmitter release in the prefrontol cortex (PFC) and the hippocampus respectively (Popoli, *et al.*, 2012).

3.2. Implications on Neurotransmitters within the Hippocampus

The hippocampus is a structure that lies deep in the limbic area of the brain which plays a pivotal role in learning, spatial and navigational memory (McEwen, 2012). It comprises of the dentate gyrus, the *cornus ammonis* (CA) 1, CA2 and CA3 layers, with each contributing to the facilitation of learning and memory in the brain (McEwen, 2012). The hippocampus has been shown to be more susceptible to neuronal insult in early life due to a high concentration of glucocorticoid receptors present in the CA1 and CA3 pyramidal neurons (Altman, *et al.*, 1990; McEwen & Sapolsky, 1995; Sapolsky, 2003). These neurons are particularly important to the hippocampus as they are responsible for plasticity that involve long-term synaptic potentiation (LTP) and depression (LTD), dendritic remodelling and neurogenesis

(McEwen, 2001). LTP and LTD are mechanisms involved in synaptic plasticity that influences neuronal changes whereby LTP promotes increased neuronal signalling resulting in enhanced synaptic strength and LTD decreases neuronal signalling thereby suppressing synaptic strength (McEwen, 1999; McEwen, 2001). This is particularly involved in consolidating short-term spatial memory to long-term spatial memory in the hippocampus (Riedel, *et al.*, 2003). The HPA-axis is closely related to the functioning of the hippocampus in which adrenal steroids play a crucial role in hippocampal formation (McEwen, 2001). Adrenal steroids influence LTP and LDP during neuronal response to stresses by acting with excitatory amino acids in the regulation of neurogenesis entailing the remodelling of dendrites in the CA3 region of the hippocampus (McEwen, 1999). Resulting in consequences often contributing to cognitive impairments namely learning and memory impairment observed in prenatally stressed rodents during behavioural tests (McEwen, 2001). Studies have shown that exposure to prolonged stress periods stunts growth and brunching of dendrites resulting in a decrease in synaptic plasticity (Hunter & McEwen, 2013). Subsequently leading to altered behaviour and cognitive impairments (McEwen, 2000; Hunter & McEwen, 2013). The cognitive impairments may be assessed in animal models using various behavioural testing methods *viz.* radial arm maze, novel object recognition test and the Morris water maze, to name a few (Morris, 1984; D'Hooge & De Deyn, 2001). While a measure of anxiety levels in a stress animal model can be assessed using the light/dark box, open field test and elevated plus maze (Cannazziro, *et al.*, 2006; Qulu, *et al.*, 2012).

A study by Martin & Wellman (2011), has shown that the involvement of glutamate and various glutamatergic receptors are responsible for the retardation of dendritic growth and branching. Excitatory amino acids particularly are important factors of the CA3 region of the hippocampus (McEwen, 1999). The excitatory amino acids are regulated by adrenal steroids activated by disturbance in the HPA-axis (McEwen, 1999). Adrenal steroids possess both rapid and delayed effects which include non-genomic mechanisms (facilitated by membrane receptors), indirect genomic mechanisms (facilitated by membrane receptors and secondary messengers) and genomic mechanisms (facilitated by cytoplasmic receptors that has a direct impact on the nucleus and act as transcriptional factors) (Popoli, *et al.*, 2012).

High concentrations of glucocorticoid activation has been shown to result in increased intracellular calcium ions, thereby exacerbating glutamate release within the hippocampus potentially causing excitotoxicity (Popoli, *et al.*, 2012). In addition, GABA and serotonin are other neurotransmitters also involved in alterations in plasticity (McEwen, 2000). Various levels of adrenal steroids have varying effects on the GABAa receptors in the CA3 region of the pyramidal cells (McEwen, 1999). Therefore GABA has been thought to play a particularly important role in neuronal excitability (McEwen, 1999). Studies have reported GABAergic transmission are calcium dependent and are also responsible for LTP (Baroncelli, *et al.*, 2011). However, emerging studies show the inter-relationship between glutamate and GABA in maintaining balance in synaptic transmission thereby maintaining neuronal plasticity (McEwen, 1999; 2001; 2008; Popoli. *et al.*, 2012).

In the presence of adversities such as stress, seizures and brain trauma, a shift in the HPA-axis occurs resulting in the imbalance between neurotransmitters glutamate/GABA and atrophy which are regarded as common underlying factors that impair hippocampal plasticity (McEwen, 2000; Damaudery, *et al.*, 2007). However, a study by McEwen (2000) showed that repetitive exposure to stressors often does not disrupt hippocampal function, instead promote neurogenesis or improved plasticity. Whereas exposure to early life stress that does not result improved plasticity, has been linked to epigenetic and neurotropic modifications that allow the hippocampus to become more vulnerable to neuronal insults (Damaudery, *et al.*, 2007).

3.3. Role of Brain-Derived Neurotrophic factor (BDNF)

BDNF, a neurotrophic factor, is a member of the neurotrophin family and is related to growth factors (Murray & Holmes, 2011). It functions to support neuronal survival by promoting dendritic spine re-organization, synaptic plasticity and neurogenesis particularly in the hippocampus (Murray & Holmes, 2011; Gray, *et al.*, 2013). Studies have established that BDNF plays a crucial role in glutamatergic and GABAergic synaptic maturation and LTP in neuronal activity (Gray, *et al.*, 2013).

BDNF is first translated as a precursor protein (proBDNF) that is proteolytically cleaved to form mature BDNF which functions upon binding to tropomyosin-related kinase B (TrkB) receptors thereby activating various signalling pathways (Gray, *et al.*, 2013). TrkB may also be activated by glucocorticoids, thus in presence of stress, there are increased levels of glucocorticoids which surpass the neuroprotective mechanism of BDNF subsequently leading to cell death in the hippocampus (Gray, *et al.*, 2013). The pathway in which glucocorticoids exerts its effect on BDNF production has not been clearly established (Rothman & Mattson, 2014).

Furthermore, the role of BDNF involving the glutamatergic and GABAergic systems has been shown to be directly involved in synaptic plasticity in which a positive feedback loop is present between the pre- and post-synaptic termini that are responsible for the generation of LTP (Murry & Holmes, 2011). Studies have shown that increased levels of intracellular calcium as a result of elevated NMDA receptor activation, suggest that BDNF is a retrograde messenger that is responsible for producing regressive action potentials (Murry & Holmes, 2011). These action potentials modulate short- and long-term changes in synaptic activity and “knocks off” the magnesium ion from resting NMDA receptors resulting in the dendritic release of BDNF thereby promoting a support mechanism for LTP and synaptic plasticity (Murray & Holmes, 2011). Therefore, in general it can be postulated that increased BDNF levels increases excitability by increasing synaptic plasticity, however, after a febrile seizure, exacerbated levels of BDNF can lead to excitotoxicity resulting in cell death (Murray & Holmes, 2011). Furthermore, BDNF regulates the signalling pathway (via the Trk pathway) between excitatory pyramidal cells and inhibitory interneurons to maintain stability in neuronal circuitries particularly under compromised conditions such as seizures (Cunha, *et al.*, 2010). This form of autoregulatory

homeostasis plays a critical role in plasticity that contributes to cognitive function (Cunha, *et al.*, 2010). Although this link is poorly understood, emerging evidence suggest that cognitive malfunction may be strongly related to alterations in the nuclear DNA-histone chromatin complex in genes that are important in gene transcription in learning and memory (Cunha, *et al.*, 2010).

4. Animal models of febrile seizures and prenatal stress

In order to mimic the human condition of febrile seizures in animal models, many resort to the use of rodent models particularly to study the pathophysiology involved in febrile seizures (Heida & Pittman, 2005; Riazi, *et al.*, 2010; Zhou, *et al.*, 2014; Suchomelova, *et al.*, 2015; Yagoubi, *et al.*, 2015). The common strain of rodent species used to mimic the febrile seizure model have been Sprague-Dawley or Wistar rats (Heida & Pittman, 2005; Riazi, *et al.*, 2010; Zhou, *et al.*, 2014; Suchomelova, *et al.*, 2015; Yagoubi, *et al.*, 2015).

There are various methodologies that have emerged to induce a febrile seizure; ranging from administration of chemoconvulsant drugs, electrical stimulation, heated water bath or heated stream of air (Heida & Pittman, 2005; Riazi, *et al.*, 2010; Zhou, *et al.*, 2014; Suchomelova, *et al.*, 2015; Yagoubi, *et al.*, 2015). The study undertaken by Dubé and colleagues (2005) used a febrile seizure paradigm of heated streamed air. Briefly, 14 and 15 day old rats were placed in glass containers and hyperthermia was induced at 41˚C using regulated heated stream of air. After 2 minutes, this method would subsequently trigger an onset of a febrile seizure (Dubé, *et al.*, 2005). While Yagoubi and colleagues (2015), placed 11 day old rats in glass bottles which were subsequently placed in 45-50˚C water bath for 30 minutes until the rats core temperature exceeded 39.5˚C. Thereafter, upon observation of myoclonic jerks, the rats were return to room temperature in which a febrile seizure would follow. Furthermore, Dai, *et al.* (2014) established a complex febrile seizure model with the use of pentylenetetrazol to induce a fever followed by maximal electroshock that evoked a seizure. Although these models seem robust, each are assessing different pathophysiologies of the various types of febrile seizures.

The model of complex febrile seizures as seen by Heida, *et al.* (2009) is a model of particular interest. To induce febrile seizures in 14 day old rats, a combination of lipopolysaccharide and kainic acid was used (Heida, *et al.,* 2009). The reason for using this combination of drugs was that the observations made from the use of pentylenetrazol, a $GABA_A$ antagonist and pilocarpine, a muscarinic cholinergic agonist, induced prolonged phases of hypothermia in the absence of lipopolysaccharide. Therefore, kainic acid, when administered alone was absent of hypothermia but produced an adequate inflammatory response (Heida, *et al.,* 2009). After considerable research on the dose dependent curve that would provide febrile seizures in atleast 50% of animals without recurrent seizures, a final dose of 200µg/kg of lipopolysaccharide administered intraperitoneally followed by 1.75mg/kg of kainic acid was adapted for this model (Heida, *et al.,* 2009). This model provides clinical relevance regarding its

temperatures and seizure duration of at least 60 minutes and is therefore considered a favourable model in many labs.

Furthermore, it is possible to mimic a prenatal stress animal model by exposing pregnant rats in their last trimester of pregnancy (gestational day 14-21) to various forms of stressors (McEwen, 2001; Moisiadis & Matthews, 2014a). These forms of stressors may vary from food deprivation, foot-shock, rodent restrainers or administering increased doses of exogenous glucocorticoids directly into the maternal circulation have proved to be an effective method to mimic a prenatally stressed rodent model (McEwen, 2001; Moisiadis & Matthews, 2014a).

To assess learning and memory in rodent animal models, as previously mentioned, various behavioural tasks may be performed *viz.* radial arm maze, novel object recognition test and the Morris water maze, to name a few (Morris, 1984; D'Hooge & De Deyn, 2001). These tasks entail exposing an animal to a training period in the behavioural apparatus to allow the animal to become familiar with the environment (Morris, 1984; D'Hooge & De Deyn, 2001). Followed by a period with no training, a test is followed to be able to assess if the animal was ble to recall the navigational and spatial memories formed from exposure to the training period (Morris, 1984; D'Hooge & De Deyn, 2001).

While a measure of anxiety levels in a stress animal model can be assessed using the light/dark box, open field test and elevated plus maze (Cannazziro, *et al.*, 2006; Qulu, *et al.*, 2012). These behavioural apparatus does not include a training period but rather lengthy test period of approximately 5-10 minutes (Cannazziro, *et al.*, 2006; Qulu, *et al.*, 2012). This time period allows the animal to overcome its novel environment and the time spent on in corners of the open field test, the dark part of the light/dark box or the closed arm of the elevated plus maze are indicators of an anxious animals (Cannazziro, *et al.*, 2006; Qulu, *et al.*, 2012). Therefore these behavioural assessments are suited to assess the anxiety-like behaviour of animals, particular those who are working with a stressed animal model (Cannazziro, *et al.*, 2006; Qulu, *et al.*, 2012).

5. Epigenetics

Epigenetics is the modifications of the chromatin network that result from external factors that lead to the alteration of gene expression without affecting the DNA sequence (Fagiolini, *et al.*, 2009). These modifications affect regulatory processes involved in CNS development, synaptic plasticity and learning and memory (Kobow & Blümcke, 2011). Memory formation requires perpetual neuronal synaptic connections (Heyward & Sweatt, 2015). These synaptic connections are often established through synapse-to-nuclear signal transduction cascades that often result in stable changes in gene expression (Adams & Sweatt, 2002). DNA-methylation is a typical underlying epigenetic mechanism that is responsible for the formation of memories in mammals (Heyward & Sweatt, 2015).

5.1. DNA Methylation

DNA methylation is associated by the formation of a covalent bond between a methyl group to the carbon situated on the cytosine pyrimidine ring (Bird, 2002). This subsequently forms a carbon-carbon bond that allows the methyl-carbon group to be highly stable (Bird, 2002). In mammal species, DNA methylation exhibits an exclusivity to binding to cytosine-phosphate-guanine (CpG) dinucleotides (Heyward & Sweatt, 2015).

DNA methylation is a process that involves various associations with DNA methyltransferases (DNMTs) post-transcriptionally to determine the stability status of gene expression (Kobow & Blümcke, 2011; Foti & Roskams, 2011). DNMTs mediate the reaction involving the binding to the methyl donor (Heyward & Sweatt, 2015). There are three conserved DNMTs in mammals; (1) DNMT3A , DNMT3B (no CpG pairs a methylated) and DNMT1 (maintains symettrical methylation of CpG pairs) (heyward & Sweatt, 2015). In addition, DNA methylation may also be associated with transcriptional suppression (Heyward & Sweatt, 2015). Transcriptional suppression occurs via direct interference with the transcription factor binding or via recruitment of transcriptional repression complexes such as methyl-CpG-binding protein 2 (MeCP2) and histone deaceylases (HDACs) (Foti & Roskams, 2011; Qureshi & Mehler, 2010; Martinowich, *et al.*, 2003; Heyward & Sweatt, 2015).

5.2. Methyl-CpG Binding Protein 2 (MeCP2)

MeCP2 is abundant in the brain particularly during embryonic development (Qureshi & Mehler, 2010). Starting as early as gestational day 14, MeCP2 is highly expressed in the brainstem (Mattarazzo, *et al.*, 2004). By gestational day 16, expression is predominant in the hippocampus and cortex suggesting its role in neuronal plasticity (Mattarazzo, *et al.*, 2004). MeCP2 is a repressor transcriptional factor that determines neuronal cell fate by associating itself with corepressor transcriptional factors; Sin3a, NCoR and c-Ski (Qureshi & Mehler, 2010). It has been suggested that MeCP2 plays a role in X-linked disorders that often result in mental retardation, motor dysfunction, cognitive impairments and various features linked to autism (Mattarazzo, *et al.*, 2004). Furthermore, studies have shown that MeCP2 is involved in neuronal maturation, cellular differentiation and synaptogenesis at critical time points in neurodevelopment (Shahbazian, *et al.*, 2002; Cohen, *et al.*, 2003; Jung, *et al.*, 2003).

The mechanism of action involving MeCP2 begins post-methylation of CpG-islands, as methylation results in the active form of MeCP2, that once formed, recruits corepressors (Qureshi & Mehler, 2010). Sin3a and HDAC 1 and 2, are some of the corepressors that once recruited by MeCP2, promotes histone deacetylation and compacting of chromatin (Qureshi & Mehler, 2010). MeCP2 directly interacts with c-terminals thereby modulating its gene expression (Mattarazzo, *et al.*, 2004).

In addition, studies have linked MeCP2 to stress and stress-associated behaviours in which it has been found that neuropeptide, corticotrophin-releasing hormone (CRH), plays a major in the HPA-axis, increases during stressful periods facilitates the increase in MeCP2 expression (Guy, *et al.*, 2011). This suggests that MeCP2 does possess neurogenic properties to overcome compromising conditions (Guy, *et al.*, 2011). Furthermore, in recent studies MeCP2 has been implicated as a gene that regulates gene transcription due to its role in contribution to seizures (Roopra, *et al.*, 2012; Qureshi & Mehler, 2010). Genomic region ubiquitin-protein ligase E (UBE3) and GABA receptor subunits are areas in which MeCP2 can exert its effect by binding to methylated gene promoters, highlighting its role in seizures due to its selective regulatory mechanisms (Qureshi & Mehler, 2010). Therefore, modifications to MeCP2 has been shown to amplify excitatory outputs leading to cognitive dysfunction and memory loss (Roopra, *et al.*, 2012). MeCP2 is also an upstream regulator of BDNF, therefore when methylation occurs at the CpG site, it suppresses all activity dependent on BDNF transcription which halts any neurogenic activity (Martinowich, *et al.*, 2003). This is seen as calcium channels are altered resulting in depolarization of the presynaptic terminal which subsequently leads to the disassociation of BDNF from MeCP2 (Martinowich, *et al.*, 2003). This subsequently results in active chromatin remodelling and transcription of altered gene expressions which is the ultimate consequence leading to neuronal dysfunction (Martinowich, *et al.*, 2003). Moreover a study conducted by Martinowich, *et al.* (2003) confirmed that DNA methylation involving MeCP2, is a major contributor to chromatin remodelling and long-term alterations in gene expressions in postmitotic neurons as it had been found that dissassociation of MeCP2 and its corepressors from BDNF after depolarization allows for active chromantin remodelling and gene activation by transcription coactivators. Another contributor associated with chromatin remodelling is the corepressor: repressor element-1-silencing transcription factor (REST) (Wu & Xie, 2006).

5.3. Repressor Element-1-Silencing Transcription (REST) factor

REST is a negative regulatory factor that is responsible for repressing gene transcription in various neural cells (Roopra, *et al.*, 2012; Wu & Xie, 2006; Griffith, *et al.*, 2001). This transcriptional factor comprises of two corepressors; CoREST and mSin3a attached to a "zinc- finger" DNA- binding domain (Wu & Xie, 2006; Griffith, *et al.*, 2001), Upon activation, the REST gene factor recruits MeCP2 and histone deacetylases resulting in chromatin remodelling (Roopra, *et al.*, 2012; Wu & Xie, 2006; Griffith, *et al.*, 2001). This is an important function of REST as it relates to the important role it plays at the level of mRNA and protein (Roopra, *et al.*, 2012). REST genes are actively transcribed in neural stem cells and progenitor cells, therefore in developing neuronal networks, REST is increased particularly in the hippocampus upon induction of a seizure (Roopra, *et al.*, 2012). This occurs because cell death resulting from seizures results in neural stem cells to “replace” the loss of cells that occur particularly in the hippocampus (Roopra, *et al.*, 2012). Thus subsequently leads to the disinhibition of the repressor gene alterating gene expression (Roopra, *et al.*, 2012; Wu & Xie, 2006). In studies conducted by Qureshi &

Mehler (2010), REST had shown to play a pivotal regulatory role in various implications in epileptogenesis, namely in growth factors, gap junctions and neurosecretory vesicles. In addition, REST is downregulated due to histone deacetylation post-exposure to kainic acid induced seizures (Qureshi & Mehler, 2010).

The MeCP2 and REST genes are important regulators of transcription particularly in processes involved in learning and memory (Martinowich, *et al.*, 2003; Roopra, *et al.*, 2012). In disease conditions such as Rett Syndrome and epilepsy, these genes have been identified as key role players to epigenetic-related cognitive dysfunction, however further research of these genes in febrile seizures are yet to be established (Martinowich, *et al.*, 2003; Roopra, *et al.*, 2012).

6. Treatments for Febrile Seizures

Conventional drugs such as carbamazepine, sodium valporate acid and phenobarbitone are amongst the common in treatment regimens in children presenting with febrile seizures (Liu. *et al*., 2015). However, adverse effects related to the use of these drugs include deep brainstem retardation, lethargy, sleep apnea and sometimes exacerbation of seizure occurrence leads to cognitive impairment (Liu. *et al*., 2015). In addition to adverse effects, in the African continent, having febrile convulsions is often associated with unnatural causes creating a stigma such that many avoid visiting local health facilities, whilst others face financial adversities resulting in many resorting to the use an alternative affordable source of treatment such as natural plant remedies (Ojewole, 2008; Glover, *et al.,* 2010; Liu. *et al*., 2015).

6.1. The potentiating therapeutic effects of *Searsia chirindensis*

In South Africa specifically, many African communities use the bark of a "Red Current" tree as a treatment and remedy to various conditions *viz.* hypertension, rheumatism and brain abnormalities (Ojewole, 2008). *Searsia chirindensis*, belongs to the semi-deciduous to deciduous Anacardiaceae family (Ojewole, 2008). Various species of this family occur throughout the globe, however *S. chirindensis* is specific to Africa, particularly in Southern Africa within the province of KwaZulu-Natal (Ojewole, 2008). This plant is a perennial species and prefers tropical to sub-tropical climates (Ojewole, 2008).

Searsia chirindensis (*Searsia*), previously called *Rhus chirindensis*, is a plant with many medicinal properties that has been traditionally used by many people in Southern Africa (Ojewole, 2008). *Searsia* also commonly known as “Red current”, belongs to the Anacardiaceae family that occurs naturally in tropical and sub-tropical regions of Africa, thus making *Searsia* easily accessible to many (Ojewole, 2007; Ojewole, 2008). Various parts of the plant are used for different conditions, for instance, the sap collected from the stem is used to treat cardiovascular complaints, whereas the bark is used to treat rheumatism and mental disorders (Ojewole, 2007). Some of the active ingredients present in the crude extract include flavonoids, which act as antioxidants; saponins that are regarded as anti-carcinogenic,

anti-oxidative and anti-diabetic; triterpenes which are regarded as precursors of steroids and possess anti-inflammatory properties as well as, tannins, a polyphenol compound which contributes to metabolism via its antioxidant properties (Ojewole, 2008). The anti-inflammatory effects of Searsia shown by Qulu, *et al.* (2015), suggests that the anti-convulsive effects of Searsia may be due to its ability to attenuate the increase in the concentration of pro-inflammatory cytokines such as IL-1β that have been shown to play a role in febrile seizure occurrence.

7. Aims

It is possible to create an animal model of febrile seizures by injecting 14 day old rat pups with 200µg/kg of the endotoxin that is part of the cell-wall in gram-negative bacteria, lipopolysacchride (Heida, *et al.*, 2009; Vasilche, 2015), thereby mimicking the inflammatory cascade as previously mentioned increasing IL-1β levels. Subsequently, this is followed by a sub-lethal dose of kainic acid (1.75mg/kg), a glutamate receptor agonist that binds at the kainate receptors, that are abundant in the hippocampus, thereby triggering an excitatory postsynaptic potential that leads to a seizure (Heida, *et al.*, 2009; Vasilche, 2015).

Therefore, the aims of this study are to investigate; 1) The role of the MeCP2 and REST genes in the developing brain by investigating the effects of early life exposure to neuronal insults (prenatal stress and febrile seizures) on learning and memory as well as determining the role of MeCP2/REST genes activation on hippocampal function. 2) Whether neuronal malformations and the resulting cognitive deficits associated with stress are linked to changes in BDNF concentration and the REST gene factor expression and whether exposure to febrile seizures exacerbates the changes seen in these factors, and 3) the effects of *Searsia* crude bark methanolic extract on prenatally stressed and febrile seizure exposed rats that exhibit cognitive impairment and whether the crude bark methanolic extract attenuates the changes observed in the hippocampus.

Chapter Two

Article 1

"Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats"

The manuscript will begin with a title, authors and affiliations on a single page. As this study was in the journal "**Neurobiology of Learning and Memory**" (Volume 125, 1 September 2015, pp 195-201) it is written according to the authors guidelines for the journal. (Addendum A).

RESEARCH PAPER

Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats.

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Abstract

Early life neuronal insults exacerbate the development of febrile seizures and can result in epigenetic changes in the hippocampus. The MeCP2 and REST genes play a pivotal role in cognition as both contribute to neuronal function. In this study, cognitive function and expression of the MeCP2 and REST genes in the hippocampus were investigated in four groups *viz.* (1) Normally reared Sprague-Dawley offspring treated with saline (NSS). (2) Prenatally stressed offspring treated with saline (SS). (3) Normally reared offspring with febrile seizures (NSFS). (4) Prenatally stressed offspring with febrile seizures (SFS). A once-off exposure to saline injections and febrile seizure induction were conducted on postnatal day (PND) 14. Pregnant dams were subjected to 1 hour of restraint stress for 7 days. Behavioural tests were conducted using the Morris-Water maze. There was a febrile seizure effect on learning and memory in the non-stressed animals. However, febrile seizures did not exacerbate learning deficits in the prenatally stressed animals. Gene analysis found a down-regulation in MeCP2 gene expression and an up-regulation of the REST gene in prenatally stressed animals. Exposure to febrile seizure resulted in down-regulation of both MeCP2 and REST gene expression in the non-stressed animals, but febrile seizures did not exacerbate the stress effect on gene expression. This suggests that exposure to prenatal stress (SS) and febrile seizures (NSFS) may impair cognitive behavioural function in the short-term. However, in the NSFS animals, there seems to be an attempt to counteract the effects of febrile seizures with time.

Keywords: Prenatal stress; febrile seizures; epigenetics; *MeCP2*; *REST*; cognitive function

1.1. Background

Febrile convulsions are a growing concern in many facets of the health fraternity within South Africa ([65];[1]). A febrile seizure may be categorised by acute episodes of synchronous firing of neurons in the brain thereby altering neuronal functional balance on the central nervous system (CNS) ([63];[51]). Febrile convulsions are shown to be exacerbated following exposure to prenatal stress ([51]).

Prenatal stress is a term used to describe the stress response experienced by a pregnant women due to various external factors (environmental factors, emotional and financial implications) thereby affecting the developing foetus ([35]). The mechanism of action of stress involves the release of glucocorticoids from the adrenal glands into the blood circulation of the expectant mother ([61];[35]). Glucocorticoids can easily cross the placental membrane under normal physiological conditions ([7];[58]). Foetal glucocorticoid concentrations are high during the last trimester, as it is required for the development of organ systems in the developing foetus ([12]). However, exposure to prenatal stress further exacerbates glucocorticoid release thereby impairing the negative feedback mechanism involving 11β- hydroxysteriod-dehydrogenase type 2 (11β-HSD2) ([12]). 11β-HSD2 is an enzyme responsible for dampening glucocorticoid effects on the developing HPA-axis in the foetus under prolonged exposure to glucocorticoids ([58];[28];[16]). Prolonged exposure to glucocorticoids has been shown to have deleterious effects on various areas of the brain in the developing foetus ([12];[40];[11]). The hippocampus is known to be more susceptible to neuronal insults in early life due to high expression of glucocorticoid receptors ([39];[57];[12]).

Prenatal stress has also been linked to changes at cellular and molecular level involving DNA methylation ([20]). DNA methylation has been shown to affect the hippocampus during development by multiple mechanisms *viz*., inhibition of DNA methyltransferases (DNMTs) resulting in reduced glutamate concentrations thereby affecting the transcriptional function of gene Methyl-CpG-binding protein 2 (MeCP2) ([53];[26]). MeCP2 is a common gene found in the brain that is susceptible to epigenetic related malfunctions particularly during the gestational, neonatal and pubertal stages of mammalian development ([52];[26]). MeCP2 functions by binding to various co-repressors (*Sin3a, NCOR,c-ski*) which activate cAMP response element-binding protein (CREB) simultaneously regulating Brain-Derived Neurotrophic Factor (BDNF) transcription ([52]). MeCP2 is a multi-functional gene (transcriptional regulation, DNA repair, chromosome segregation), which makes it susceptible

to mutation during neural development ([52];[26]). Symptoms related to MeCP2 mutations include autistic features, seizures and mental retardation ([52];[26]). Furthermore, Repressor Element-1 Silencing Transcription factor (REST) has been shown to be a pivotal player in epigenetics ([52]). Initially it was thought that REST silences neuronal gene transcription in supporting cells, however studies have emerged showing that REST is the master regulator of neurogenesis along with gene silencing ([9]). REST mediates its action of chromatin and histone modification through the recruitment of transcriptional repressors SIN3A/SIN3B and methylated CpG binding proteins; MeCP2 ([37];[6]). Upon stimulation such as depolarization, MeCP2 and other SIN3A leave the CpG site, allowing for higher level of gene expression ([6]).

Therefore in our study we aimed to better our understanding of the MeCP2 and REST genes in the developing brain by investigating the effects of early life exposure to neuronal insults (prenatal stress and febrile seizures) on learning and memory as well as determining the role of MeCP2/REST genes activation on hippocampal function.

2.1. Materials & Methods

2.1.1. Animals

Sprague-Dawley rats were obtained from the Biomedical Resource Centre (BRC) of the University of KwaZulu-Natal. The animals were kept under standard BRC conditions (20% humidity at temperature of ±30˚C) and a 12hr light/dark cycle (lights on at 06h00) was maintained. Food (Rodent Ripe Pellets, Meadow, South Africa) and water were available *ad libitium*. All animals were housed in standard conventional polycarbonate 1291H techniplast cages (425 x 266 x 185mm, floor space: 800m^2). All experimental procedures were approved by the Animals Ethics Research Committee of the University of KwaZulu-Natal (073/14/animal) in accordance with guidelines of the National Institute of Health, USA.

2.1.2. Prenatal Handling

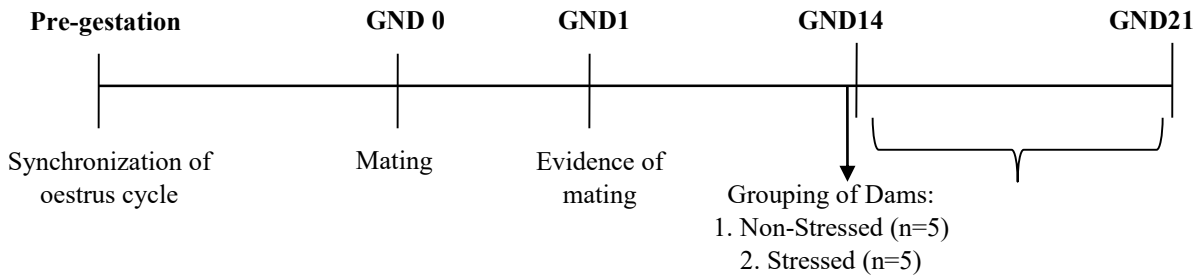

Figure 1. Outline of prenatal handling protocol

i. Synchronization

Ten female Sprague-Dawley rats were housed in pairs for a week to synchronize their oestrus cycles. The oestrus cycle of rodents is approximately four to five days, with each day representing 1 of 4 phases; pro-oestrus, oestrus, met-oestrus and di-oestrus. Each phase displays distinct cell maturity ([46];[31]). Vaginal smears were taken daily to identify the pro-oestrus phase of their cycle.

ii. Mating

A male Sprague-Dawley rat was introduced to a pair of female rats during their pro-oestrus phase. Vaginal smears were taken the following morning. The presence of sperm indicated successful mating and was regarded as gestational day 1 (GND 1). Following successful mating, the male rat was removed from the cage.

iii. Prenatal Stress Protocol

On gestational day 14 (GND 14), pregnant dams were divided into 2 groups: a non- stressed and a stressed group. The non-stressed rats were left undisturbed in their home cages. The stressed rats were taken daily to a separate room and placed in rodent restrainers for 1 hour daily for 7 days starting at 09h00. The rodent restrained rats were returned to their housing room at the end of every stress period.

2.1.3. Post-Natal Handling

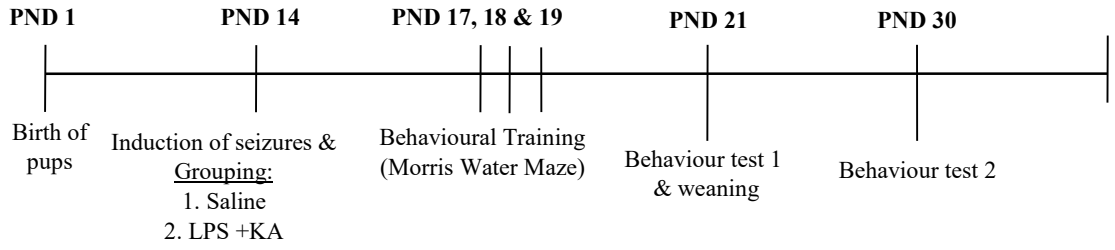

Figure 2. Outline of post-natal handling protocol

i. Birth of pups

Following birth, the pups remained with their dams undisturbed until PND 14 after which they were divided into the following groups (n=6 per group);

Table 1. Pups were grouped as follows:

Non- Stressed Animals (NS)	Stressed Animals (S)
Saline (NSS)	Saline (SS)
Febrile Seizure (LPS + KA) (NSFS)	Febrile Seizure (LPS + KA) (SFS)

The pups were removed from their dams and placed in clean cages. Each cage, housing 6 pups per experimental group was taken to the experimental room an hour prior to administration of LPS and kainic acid so as to acclimatize to the new surroundings. The dams remained in the home room, undisturbed during the duration of the experimental procedure.

ii. Induction of seizures

Control animals were injected with 2ml saline (0.9% NaCl, Adcock Ingram, South Africa) as a vehicle. To induce a febrile seizure; 0.2ml of LPS (200 µg/kg, Sigma, USA) was injected intra-peritoneally (i.p), after which pups were returned to their dams to allow for suckling and grooming. 2.5 hours later, pups were once again removed from their dams and injected with 0.2ml kainic acid (1.75 mg/kg, i.p, Sigma, USA) ([29];[51]).

a) Assessment of Febrile Convulsions

The convulsive behaviour of each rat was observed for a period of 60 minutes and the degree of convulsive behaviour was measured (Table 2) ([62];[29];[47]).

Table 2. Seizure response were scored as follows:

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

On PND 21, pups were weaned from their dams and the dams were returned to the animal unit. On PND 21, learning ability was assessed on both male and female pups- whereas the ability to remember (memory) was assessed on PND 30 male pups only. Only male pups were used on PND 30 so as to negate the effects of hormonal changes associated with female rats ([48]).

2.2.1. Behavioural Tests

Morris Water Maze

Training in the Morris Water Maze (MWM) took place between PND 17-19. This was followed by a test phase (PND 21 and PND 30) which assessed learning and memory deficits. This test is one of many which determines the learning and recall ability of rats, and covers exploratory, navigational, spatial and contextual memory ([45];[18];[23]). The MWM consists of a 1m diameter pool, comprising of 4 quadrants. Each quadrant has a cue to assist the rat in finding the hidden platform located in one of the quadrants of the pool. The method entails placing the rat in a quadrant other than where the hidden platform is located, recording the time undertaken

for the rat to reach the hidden platform is considered as the animals ability to learn ([45];[18];[23]). The probe test is a post-test for learning which measures the ability of an animal to remember the quadrant in which the hidden platform is located. The time spent in the quadrant of the hidden platform is considered as the ability to remember (memory) ([45];[18];[23]).

Training procedure

Animals were exposed to three training sessions for a period of three days (PND 17-19). Animals were taken to the behavioural room one hour prior to training to allow for acclimatization to the new environment. For the training procedure, each rat was placed in a quadrant, gently placed in the water, head facing the cue. The rat was given 120 sec to find the hidden platform. If a rat failed to find the hidden platform in 120 sec, it was physically guided by the experimenter towards the platform and was allowed 60 sec to explore the platform before being returned to the home cage. Training resumed the following day for the next two days. No training took place on PND 20.

Testing procedure

On PND 21, animals were removed from their rooms and taken to the behavioural room one hour prior to the behavioural test to allow for animals to acclimatize. Thereafter, animals were placed in a quadrant other than the hidden platform and the time to locate the hidden platform was recorded. Animals were then returned to their cages before being returned to their room. Animals were left undisturbed from PND 22-29. On PND 30, the probe test was conducted. Prior to the test, the animals underwent the same acclimatization procedure previously described. For the probe test, the platform was removed completely from the maze and the animals were allowed 120s in the water. Time spent in the quadrant of the hidden platform was timed. Following the probe test, the animals were returned to their home room. Animals were sacrificed by decapitation 5 days later (PND 35), three weeks after seizure induction.

2.2.2. Sacrifice

On PND 35, the rats were taken to the autopsy room 1 hour before decapitation. The rats were decapitated using a guillotine; trunk blood and hippocampal tissue were collected. Hippocampal tissue was weighed prior to freezing in liquid nitrogen. All tissue material was stored in a bio freezer at -80 ˚C.

2.2.3. Neurochemical Analysis

Real-Time PCR (qPCR)

Approximately 50mg of hippocampal tissue was weighed out (n=6 per group), homogenized and suspended in 400µl of lysis buffer (Zymo Research, USA). Thereafter, samples were homogenised using a sonicator and total RNA isolation was carried out as per manufacturers guidelines (ZR RNA MiniPrep^{TM}, USA). Purification of RNA isolates were conducted using a NanoDrop. Purity of 1.5-2.01 was considered ideal for use in the construction of cDNA. cDNA synthesis was done using the iScript™ cDNA Synthesis Kit (Biorad, South Africa), processing guidelines were provided by the manufacturer. cDNA was run through the Thermocycler as per conditions stipulated in the guidelines. The Fast start SYBR green kit (Roche Diagnostics, USA) was used in accordance to the manufacturers protocol.

Primer sequences; MeCP2 gene- Forward primer: CGTCCCCTTGCCTGAAGGTTGGA, Reverse primer: CTTTCCAGCAGAGCGACCAG; REST gene- Forward primer: CAGTTGAACTGCCGTGGG, Reverse primer: CATCCGCTGTGACCGCTG; Beta actin gene- Forward primer: GCTTCTTTGCAGCTCCTTCGT, Reverse primer: CCAGCGCAGCGATATCG were reconstituted in RNA nuclease free water according to manufacturers report (Inqaba) and was added to a master mix comprising of SYBR green dye, nuclease free H_2O and $MgCl_2$. Thereafter cDNA was added into glass capillaries and run in the Lightcycler 480 at optimised conditions.

2.2.4. Statistical Analysis

The data was analyzed using the software GraphPad Prism (version 5). Normality and Gaussian distribution were determined using the Smirnov-Kolmogorov tests. A two-way factorial analysis of variance was performed on behavioural and gene data with stress and febrile seizures as between rat factors. Significant main effects were followed by *Bonferonni* post hoc test. Differences were considered significant when p- value < 0.05.

3.1. Results

3.1.1. Morris Water Maze: Learning and Memory Assessment

The following groups were assessed for learning and memory deficits; non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile

seizures (SFS). In the non-stressed (NS) groups we found a febrile seizure effect, as rats that were exposed to febrile seizures took a longer time to locate the hidden platform *(NSS vs. NSFS, $F_{(1.0)} = 5.15$, $p < 0.05$).

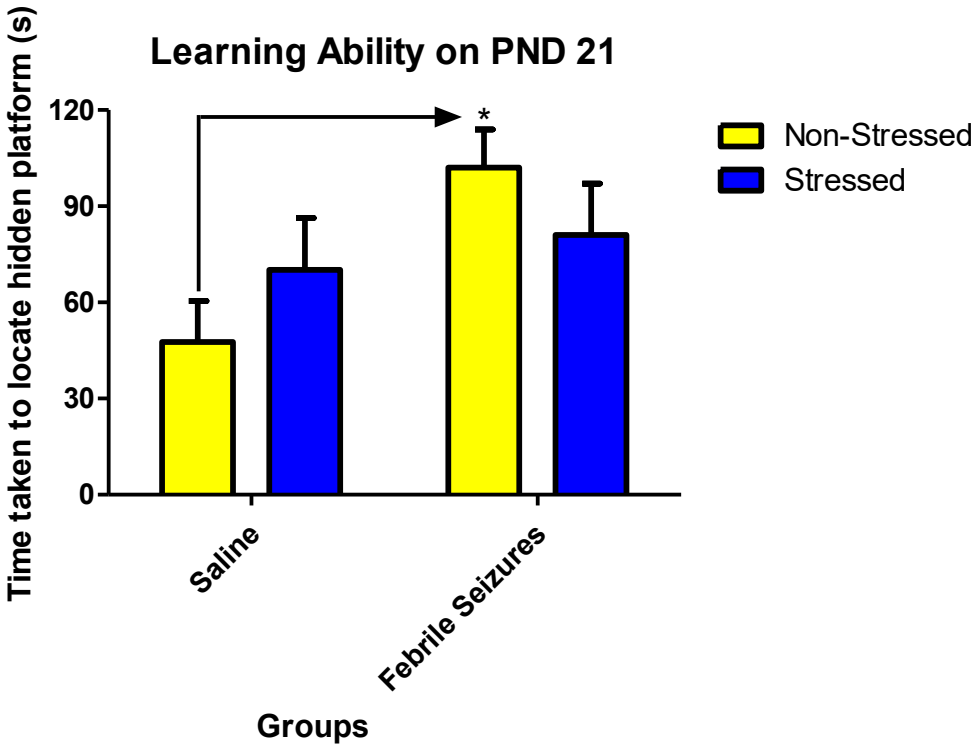

Figure 3.1.**a.** Graph displaying average time taken to locate the hidden platform in the MWM in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group.*(NSS vs. NSFS), $p < 0.05$.

A week later (PND 30) non-stressed with febrile seizure animals (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS) were once again assessed using the probe test. Our findings once again showed that in non- stressed (NS) animals there was a febrile seizure effect as febrile seizure exposed rats spent less time in the quadrant of the hidden platform *(NSS vs. NSFS, $F_{(1.0)} = 5.15$, $p < 0.05$).

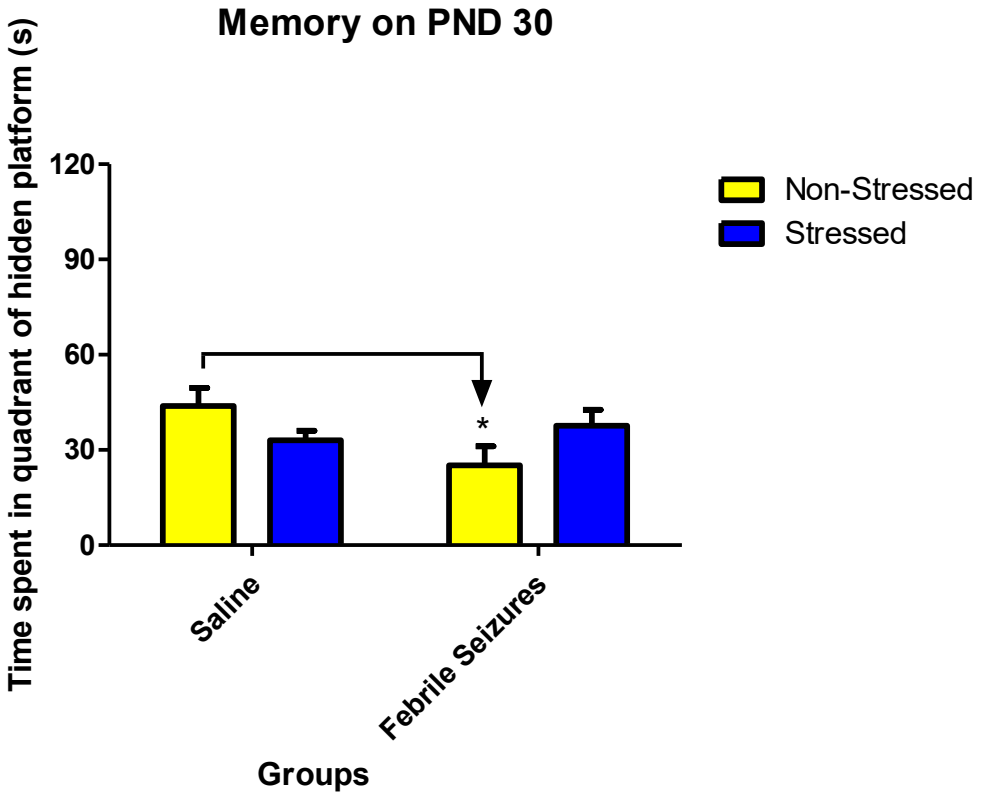

Figure 3.1.b. Graph displaying average time spent in the quadrant of the hidden platform in the MWM in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group. *(NSS vs. NSFS), $p < 0.05$.

3.1.2. RT-PCR: MeCP2 and REST genes

Real-time PCR was used to analyse the MeCP2 and REST genes. Groups analysed were non-stressed with saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS) animals. There was a stress effect as prenatally stressed rats showed down-regulation of the MeCP2 gene *(NSS vs. SS, $F_{(1.0)} = 4.93$, $p < 0.05$). There was a febrile seizure effect in the non-stressed rats **(NSS vs. NSFS, $F_{(1.0)} = 4.93$, $p < 0.05$).

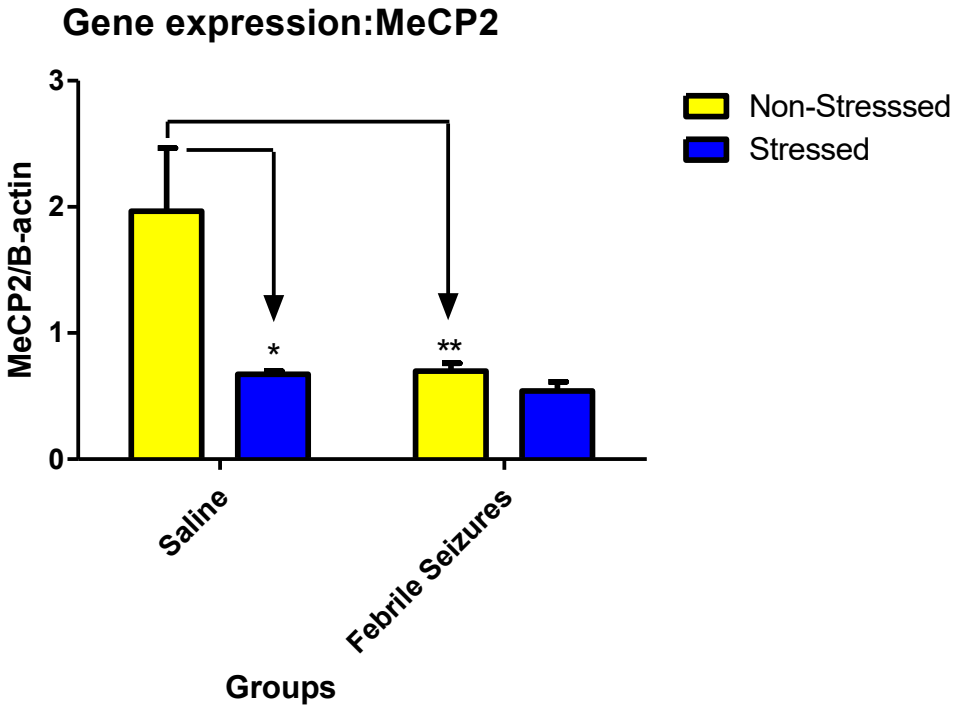

Figure 3.2.a. Graph displaying MeCP2 gene expression in the hippocampus in P30 rats in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group. *(NSS vs. SS) and **(NSS vs. NSFS), $p < 0.05$.

In REST gene analysis, there was a febrile seizure effect in non-stressed rats as rats exposed to febrile seizures showed down-regulation of the REST gene expression *(NSS vs. NSFS, $F_{(1.0)} = 2.32$, $p < 0.05$).

Figure 3.2.b. Graph displaying REST gene expression in the hippocampus in P30 rats in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group. *(NSS vs. NSFS), $p < 0.05$.

In non-stressed animals there was a significant interaction between the MeCP2 and REST genes in febrile seizure exposed animals *(NSFS MeCP2 vs. NSFS REST, $F_{(1.0)} = 14.8$, $p < 0.05$).

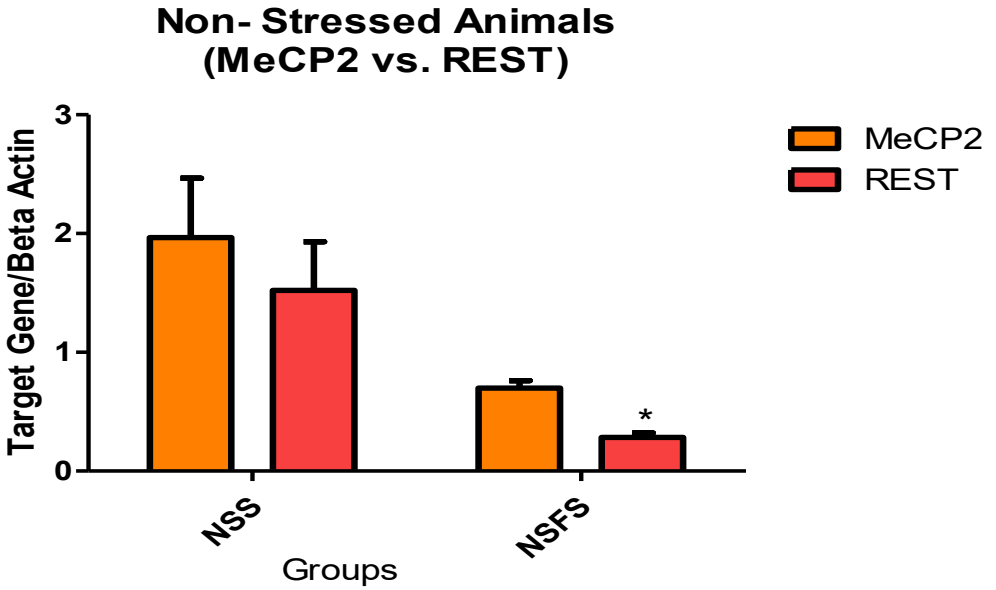

Figure 3.3.a. Graph displaying a comparative analysis of overall gene expression in non-stressed (NSS) and non-stressed febrile seizure (NSFS), n=6 per group. *(NSFS MeCP2 vs. NSFS REST), $p < 0.05$.

In stressed animals there was a significant interaction between the MeCP2 and REST genes in saline and febrile seizure exposed animals *(SS MeCP2 vs. SS REST, $F_{(1.0)} = 3.23$, $p < 0.05$) and *(SFS MeCP2 vs. SFS REST, $F_{(1.0)} = 3.23$, $p < 0.05$).

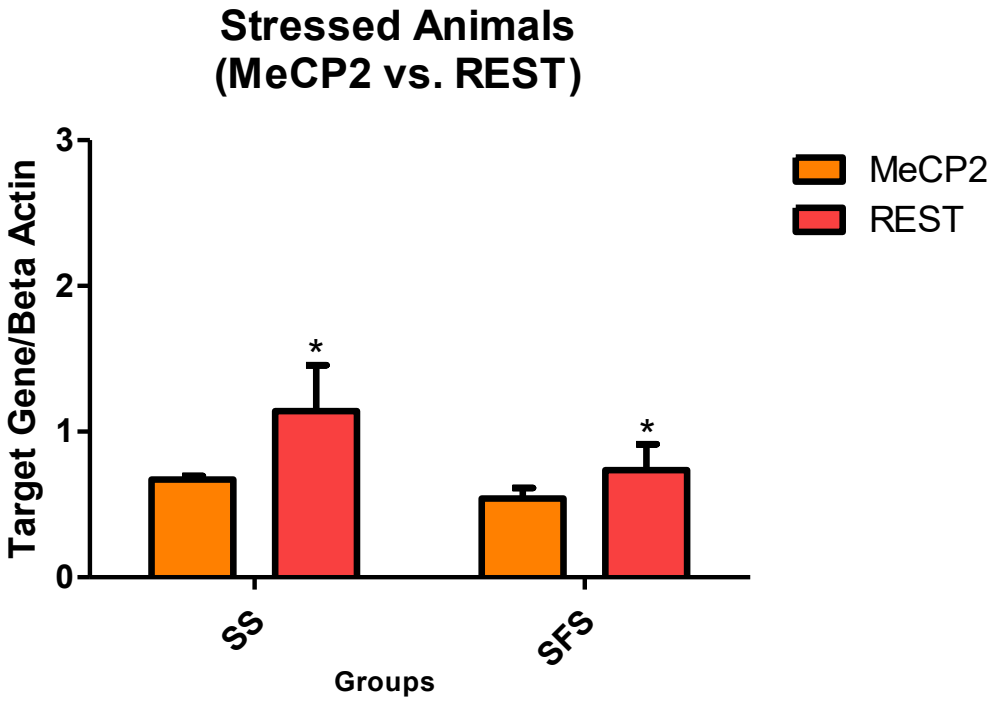

Figure 3.3.b. Graph displaying a comparative analysis of overall gene expression in stressed (SS) and stressed febrile seizures (SFS), n=6 per group. *(SS MeCP2 vs. SS REST) and *(SFS MeCP2 vs. SFS REST), $p < 0.05$.

4.1. Discussion

In our study we investigated the effects of stress on learning and memory and whether any effects present are influenced by the expression of the MeCP2 and REST genes. We also investigated whether exposure to febrile seizures may influence learning and memory behaviour as well as gene expression.

Exposure to early life stress (SS) did not seem to affect learning and while there was a tendency for the prenatally stressed (SS) rats to struggle to find the quadrant of the hidden platform, the stress effect was not significant, however a febrile seizure effect was present in the non-stressed (NSS) animals. This effect was prolonged as rats tested 15 days post febrile seizure induction also showed a presence of memory deficits. This suggests that there are learning and memory deficits present in non-stressed animals exposed to febrile seizures (NSFS). These findings are

in agreement to literature that showed exposure to multiple forms of neuronal insults did not affect cognitive function in the developing brain, however there was a degree of impairment observed in adult rats that were exposed to early life multiple episodes of a febrile seizures ([14]). In our study we were able to observe cognitive impairment with a single episode of a febrile seizure. These findings may be related to the neuroinflammation present in the brain due to the presence of a fever which triggers a seizure. Studies have shown that high levels of cytokine expression directly affects neuronal function *viz.* long-term potentiation, glutamate release and activation of cell-signalling pathways ([43];[21]). These are related to synaptic plasticity and are therefore important in cognition. Previous studies have shown that the occurrence of a fever resulted in the release of various inflammatory markers ([53];[22]). A common laboratory method to mimic a model of inflammation via fever is by injecting lipopolysaccharide (LPS), a gram negative bacterial endotoxin that triggers the activation of innate immunity via toll-like receptors (TLRs) promoting transcription of pro-inflammatory (IL-1β, IL-6, TNFα) and anti-inflammatory (IL-1 receptor antagonist (ra), IL-10) cytokines ([34];[21]). A study showed that peripheral administration of LPS resulted in long-term exposure to high levels of the pro-inflammatory cytokine IL-1β in circulation leading to compromised neural function in the brain ([49]). Learning and memory impairments have been reported in studies presenting acute exposure to various forms of peripheral inflammation which resulted in prolonged brain excitability ([53];[64]). This subsequently led to excess release of intracellular Ca^{2+} due to competitive binding between IL-1β and IL-1ra leading to neuronal cell death and compromised cognitive function ([53];[48];[64]).

On PND 21, animals exposed to stress and febrile seizures (SFS) showed no significant learning deficits from stressed animals without febrile seizures (SS), however both stressed groups (SS and SFS) had a tendency to struggle to find the quadrant with the hidden platform when compared to the non-stressed animals. The lack of difference in time taken to find the quadrant with the hidden platform in stressed animals induced with febrile seizures (SFS) suggests that the febrile seizure did not exacerbate learning impairments that may have been present. This was also the case on PND 30 as we could not find differences in the recall ability of the stressed animals (SS and SFS) when compared to the non-stressed animals without exposure to febrile seizures (NSS).

Various studies have shown that early life neuronal insults can result in chronic physiologic and neuronal alterations in later life ([60];[27];[21]). Findings from our behavioural results were confirmed by gene analysis performed on hippocampal tissue on PND 35. We

investigated the roles of the MeCP2 and REST genes in learning and memory in a prenatally stressed febrile seizure rat model.

We showed that the MeCP2 gene was significantly down-regulated in prenatally stressed animals (SS) in comparison to non- stressed animals (NSS) thereby confirming the presence of a stress effect. While we could not confirm definitive impairment in the behavioural tests, possibly due to the number of animals tested, the gene expression results were more robust in showing the presence of a stress effect. This suggests that prenatal stress had a direct effect on the epigenetic modification in the developing brain. Studies have shown the deleterious effects of various stressors in early life on brain development ([56];[39];[40];[41]). Early life stress can cause selective methylation or demethylation on specific cytosine residues in the DNA sequence which are shown to be influenced by 11β-HSD2 levels i.e. decrease in MeCP2 levels results in the decrease of 11β-HSD2 enzyme, resulting in compromised brain function in the foetus ([4]; [67]). Therefore our findings suggest that prenatal stress exposure alone has deleterious effects on the developing brain which can result in long-term learning and memory deficits.

Non-stressed animals exposed to febrile seizures (NSFS) showed a significant down-regulation of the MeCP2 gene when compared to control animals (NSS) thereby confirming a febrile seizure effect. These results may be related to spontaneous excitatory activity that occurred in early life exposure to an acute seizure. A study showed that a decrease in MeCP2 gene is due to a decrease in excitatory postsynaptic currents (EPSCs) ([15]). This was also presented in another study that showed the MeCP2 gene regulates the amount of glutamate receptors in hippocampal neurons ([13]). In our study, the acute bout of excitability, due to the injection of the kainic acid, may have resulted in over-excitation of glutamate receptors. The injection of this glutamate receptor agonist may have influenced the down-regulation of the MeCP2 gene.

Stressed animals exposed to febrile seizures (SFS) did not exhibit learning and memory deficits when compared to control animals (SS) suggesting that exposure to febrile seizures did not exacerbate the MeCP2 gene down-regulation. A study showed that stress increased arginine vasopressin in the hypothalamus which significantly decreased MeCP2 gene expression ([45]). We found no change in MeCP2 expression between stressed animals with and without febrile seizure induction (SS and SFS). Therefore, exposure to a single early life stress may impair cognition but further exposure to infections resulting in a febrile seizure does not exacerbate these impairments.

The REST gene regulates its expression through recruitment of various co-repressors including MeCP2 ([37];[6];[52]). Therefore if MeCP2 expression increases, REST will decrease. As MeCP2 gene promotes neurogenesis and the REST gene acts as a repressor factor, an increase in the MeCP2 gene expression acts as a protective feedback mechanism ([52]). Our findings have shown that there was significant up-regulation in the REST gene expression in stressed animals (SS), suggesting compromised neural plasticity. This concurs with our behavioural result in which stressed animals showed tendencies of learning and memory impairments. A study suggested that decreased expression of REST gene promotes cellular differentiation and neurogenesis ([19]). While we did not measure neurogenesis in our study, we postulate that an increase in REST gene expression affected plasticity thereby compromising hippocampal function leading to learning and memory deficits.

A febrile seizure effect was present in non-stressed animals (NSFS) as there was a significant down-regulation of the expression of the REST gene. As behavioural results showed impaired cognitive function in these animals, down-regulation of REST gene suggests that the hippocampal damage leading to cognitive impairment in these animals may be a transient effect. Which we may confirm that febrile seizures are indeed a benign condition that does not lead to permanent cognitive deficits. In order to create a febrile seizure animal model, animals were exposed to lipopolysacchiride and kainic acid. Kainic acid, a glutamate agonist, promotes increased intracellular Ca^{2+} concentrations, leading to excitotoxicity causing neuronal loss ([37]). Studies have previously shown that glutamate plays a role in regulation of REST gene expression and that early life neural insults affect glutamate receptors by desensitizing the receptors after prolonged exposure to glutamate thereby compromising hippocampal cell survival ([54];[2]). These changes in expression of MeCP2 and REST genes also showed a significant interaction suggesting that the relationship between both genes contributes to changes in hippocampal functioning. A study showed the inter-dependent relationship of the MeCP2 and REST genes suggesting that REST does silence MeCP2 under compromised conditions ([37]).

Stressed animals that were exposed to febrile seizures (SFS) showed no significant changes in the REST gene expression. This concurs with behavioural findings and MeCP2 gene expression results. We suggest that the damage caused by exposure to prenatal stress was great enough in hippocampal neurons, such that further exposure to a toxic insult did not further exacerbate the damage.

5.1 Conclusion

Our findings suggest that exposure to febrile seizures early in life affects learning and memory ability. However, it seems as if these cognitive deficits are transient as gene expression results suggests that there may be an attempt to promote neural plasticity following the febrile seizure episode. We also showed that exposure to stress early in life may affect the ability to promote neural plasticity in the hippocampus. This effect is, however, not exacerbated by further exposure to infections that lead to the development of febrile seizures.

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Disclosure

We hereby disclose that there are no known financial and personal conflict of interests in the work of this project.

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Chapter Three

Article 2

"Febrile seizure induction impairs neuronal plasticity in prenatally stressed rat offspring"

The manuscript will begin with a title, authors and affiliations on a single page. As this study will be in the journal "**Annals of Neuroscience**" it is written according to the authors guidelines for the journal. (Addendum B).

RESEARCH PAPER

Febrile seizure induction impairs neuronal plasticity in prenatally stressed rat offspring.

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Abstract

Febrile seizures in young children are a growing concern in developing economies around the world. Although this condition is regarded as benign, exposure to early life stress in these children may affect neuronal functioning in due time. We investigated whether neuronal malformation and the resulting cognitive deficits associated with stress are linked to changes in BDNF and the REST gene factor concentration and expression respectively. Furthermore, we investigated whether febrile seizure induction exacerbates the changes seen in these factors. Febrile seizures were induced on postnatal day (PND) 14 in pups of dams that were stressed during pregnancy. To assess the effects of prenatal stress on behaviour, behavioural assessments were conducted on PND 21 followed by sacrifice by decapitation. Our results show that there was a delay in the onset of seizures followed by a prolonged seizure duration in animals exposed to prenatal stress. Furthermore, prenatal stress exposure resulted in anxiety-like behaviour in young rats. We also found that prenatal stress resulted in increased hippocampal BDNF concentration, whereas exposure to febrile seizures increased REST gene expression in prenatally stressed animals. These findings suggest that exposure to prenatal stress impedes neurotrophic factor release, while accentuating the REST gene expression in febrile seizure animals. These factors may contribute to the hindering of neurogenic properties in the young, thus leading to neuronal plasticity deficits and possibly cognitive malfunction in later life.

Keywords: Anxiety-like behaviour, REST gene, BDNF, prenatal stress, febrile seizures, neural plasticity

1.1 Background

Elevation in body temperature often follows the presence of an infection in the body (Heida, *et al.*, 2004; Wager, *et al.*, 2009; Ackermann & Van Toorn, 2012). In children, infections are common triggers of seizures (Heida, *et al.*, 2004; Ackermann & Van Toorn, 2012; Wager, *et al.*, 2014). Seizures are categorised as epileptic (disorder of brain function that trigger episodic and unpredictable seizure occurrence) or non-epileptic (once-off seizure that may be evoked by electrical or chemical stimulations (McNamara, 1994). Febrile seizures are regarded as non-epileptic seizures and may be classified as a simple, complex or generalized convulsion (McNamara, 1994). Clinical findings suggest that non-epileptic convulsions lead to motor disorientation and transient cognitive deficits in patients (Hoefnagels, *et al.*, 1991). Stress has been shown to aggravate seizure progression in a kainic acid induced seizure rodent model (Qulu, *et al*., 2012). More than 10% of children who had previously been exposed to various forms of early life stressors had exacerbated seizure occurrences (Wood, *et al*., 2004). Clinical findings have further suggested that early exposure to stress affects anxiety levels thereby decreasing immune function resulting in children being more susceptible to febrile convulsions (Koh, *et al.*, 1998; Ackermann & Van Toorn, 2012).

Furthermore, it has been shown that exposure to various forms of stressors prenatally, affects the hypothalamic-pituitary-adrenal (HPA) axis particularly of the developing foetus (Qulu, *et al.*, 2012; Moisiadis, *et al.*, 2014). As a consequence, the developing neuronal circuitries in the foetus are affected due to exposure to high concentrations of circulating maternal glucocorticoids (Wilson, *et al.*, 2013; Moisiadis, *et al.*, 2014). This subsequently affects the limbic area of the brain, specifically the hippocampus, as there is a high prevalence of cortisol receptors present (McEwen, 2001; Wilson, *et al.*, 2013). Therefore, disruption in hippocampal function contributes to various anxiety disorders and cognitive deficits (McEwen, 2001; Wilson, *et al.*, 2013; Moisiadis, *et al.*, 2014).

Qulu, *et al.* (2015), has shown that prenatal stress reduces hippocampal mass in young rats. This may be a result of neuronal malformation involving various factors that involve disruption in DNA methylation affecting neurotrophic factor concentrations and multiple down- and up-stream signalling pathways altering neuronal function and structure (Qureshi & Mehler, 2010; Guy, *et al.*, 2011). Brain-derived neurotrophic factor (BDNF), a crucial player involved in neuronal plasticity, may result in the manifestation of various neuronal abnormalities and disorders when compromised (Karpova, 2014). BDNF has been closely associated with

epigenetic factors involved in DNA methylation (Karpova, 2014). Although methyl-CpG-binding proteins actively manipulate the function of BDNF, RE1-silencing transcriptional (REST) factor is essentially required to regulate transcription suggesting its involvement in neurogenesis (Pruunsild, *et al.*, 2011). It has been shown that febrile seizures have a transient effect on cognitive function by altering the balance between methyl-CpG-binding proteins and the REST gene factor in the long term (Cassim, *et al*, 2015).

Therefore the aim of this study was to investigate whether neuronal malformations and the resulting cognitive deficits associated with stress are linked to changes in BDNF concentration and the REST gene factor expression. We further investigated whether exposure to febrile seizures exacerbates the changes seen in these factors.

2.1. Material and Methods

2.1.1. Animals

Male and female Sprague-Dawley rats were obtained from the Biomedical Resource Centre (BRC) of the University of KwaZulu-Natal. The animals were housed under standard BRC conditions (20% humidity at temperature of ±23˚C) and a 12 hour light/dark cycle (lights on at 06h00) was maintained. Food and water were available *ad libitium*. All experimental procedures were approved by the Animal Ethics Research Committee of the University of KwaZulu- Natal (073/14/animal) in accordance with guidelines of the National Institute of Health, USA.

2.1.2. Prenatal Handling

a. Mating

Ten female Sprague-Dawley rats were housed in pairs for a week to synchronize their oestrus cycles (Nelson *et al*., 1982; Hubscher *et al.*, 2005). Vaginal smears were taken daily to identify when the rats were in pro-oestrus (Shorr, 1941; Moosa, *et al.*, 2014). When females reached pro-oestrus, a male rat was introduced to a pair of females, followed by the taking of vaginal smears the next morning. The presence of sperm indicated a successful mating and was regarded as gestational day (GND) 1. Following successful mating, the male rat was removed from the cage.

b. Prenatal Stress Protocol

On GND 14, pregnant dams were divided into 2 groups: a non-stressed and a stressed group. The non-stressed rats were left undisturbed in their home cages. The stressed rats were taken daily to a separate room and placed in rodent retainers for 1 hour, for 7 days starting at 09h00. The rodent restrained rats were returned to their home room at the end of every stress period (Qulu, *et al.*, 2012; Cassim, *et al*., 2015).

2.1.3. Post-Natal Handling

Following birth, the pups remained with their dams undisturbed until post-natal day (PND) 14, after which they were divided into the following groups (n=6 per group per cage);

a. Non-stressed saline (NSS)
b. Prenatally stressed saline (SS)
c. Non-stressed febrile seizure (NSFS)
d. Prenatally stressed febrile seizure (SFS)

The experimental protocol was performed in the same way as mentioned by Cassim, *et al.* (2015). Briefly, control animals were injected with 0.2 ml/g saline (0.9% NaCl, Adcock Ingram, South Africa) as a vehicle. To induce a febrile seizure (groups c and d); 0.2 ml/kg of LPS (200 µg/kg, Sigma, USA) was injected intra-peritoneally (i.p) and pups were returned to their dams to allow for suckling and grooming to avoid dehydration and hypothermia. Two and a half hours later, pups were once again removed from their dams and injected with 0.2 ml/g kainic acid (1.75 mg/kg, i.p, Sigma, USA) to induce a febrile convulsion (Heida, *et al*., 2004; Qulu, *et al.*, 2012; Cassim, *et al.*, 2015). The convulsive behaviour of each rat was observed and recorded for a period of 60 minutes. The various stages of convulsive behaviour were assessed following the guidelines provided in Heida, *et al.* (2004) and Ojewole (2008). Thereafter, pups were returned to their dams.

2.2.1. Behavioural Test

Elevated Plus Maze (EPM)

The elevated plus-maze (EPM) is a test used to assess the anxiety-like behaviour of an animal exposed to various stressors (Cannazziro, *et al.*, 2006). On PND 21, the rats were assessed for

anxiety-like behaviour using the elevated plus maze (Cannazziro, *et al.*, 2006). The test entails placing the rat in the central area of the maze facing the open arm and the degree of anxiety-like behaviour is measured by the number of entries into the preferred arm (Cannazziro, *et al.*, 2006). Animals were allowed 5 minutes in the apparatus and preference for the closed arm was regarded as a measure of anxiety-like behaviour. An entry was scored when all four paws entered into each arm.

2.2.1. Sacrifice

Rats were sacrificed by decapitation. Hippocampal tissue was collected and weighed prior to freezing in liquid nitrogen. All tissue material was stored in a bio-freezer at -80 ˚C.

2.2.3. Neurochemical Analysis

Real-Time PCR (qPCR)

The qPCR analysis was done as previously described (Cassim, *et al.*, 2015). Briefly, hippocampal tissue (50mg) was homogenized and suspended in 400µl of lysis buffer (Zymo Research, USA). This was followed by RNA extraction using the guidelines stipulated by the manufacturer (ZR RNA MiniPrep^{TM}, USA). Purification of RNA isolates was conducted using a NanoDrop. Purified RNA isolates were used in conjunction with the iScript™ cDNA Synthesis Kit (Biorad, South Africa), to construct cDNA using a Thermocycler as per conditions stipulated by the manufacturer. The Fast start SYBR green kit (Roche Diagnostics, USA), cDNA and primers were used to amplify our gene of interest at optimised conditions in the Lightcycler 480 (Roche Diagnostics, USA).

The primer sequences used:

Primer sequences;

a. REST gene-
   Forward primer: CAGTTGAACTGCCGTGGG,
   Reverse primer: CATCCGCTGTGACCGCTG;
b. Beta actin gene-
   Forward primer: GCTTCTTTGCAGCTCCTTCGT,
   Reverse primer: CCAGCGCAGCGATATCG
   (Inqaba, South Africa).

2.2.4. Immunoassay (ELISA)

BDNF protein was measured with a conventional enzyme-linked immunosorbent assay (ELISA) system. The BDNF immunoassay (Cusabio Biotech Co., Ltd., China) was performed according to the manufacturer's protocol. The harvested hippocampal tissue was rinsed and stored in 1 ml of PBS overnight at -20°C. After two freeze thaw cycles, homogenates were centrifuged at 5000xg at 7°C. The supernatant was used to determine the BDNF concentration. The sensitivity of the assay was 0.312 ng/ml- 20 ng/ml, and the cross-reactivity with other related neurotrophic factors was minimum. The intra- and inter-assay precisions were <10%, respectively.

2.2.5. Statistical Analysis

The data was analyzed using the software GraphPad Prism (version 5). Normality and Gaussian distribution were determined using the Kolmogorov-Smirnov test. A two-way factorial analysis of variance was performed with stress and febrile seizures as between rat factors. Significant main effects were followed by the *Bonferonni* post hoc test. Differences were considered significant when p- value < 0.05.

3.1. Results

3.1.1. Onset of seizure and seizure duration

Seizures were induced on PND 14 in non-stressed (NSFS) and stressed (SFS) animals. There was a stress effect as animals exposed to prenatal stress exhibited with a delay in seizure onset but had seizures of prolonged duration *(NSFS onset of seizure vs. SFS onset of seizure, $F_{(1.0)} = 132.2$, $p < 0.05$) and **(NSFS seizure duration vs. SFS seizure duration, $F_{(1.0)} = 32.6$, $p < 0.05$).

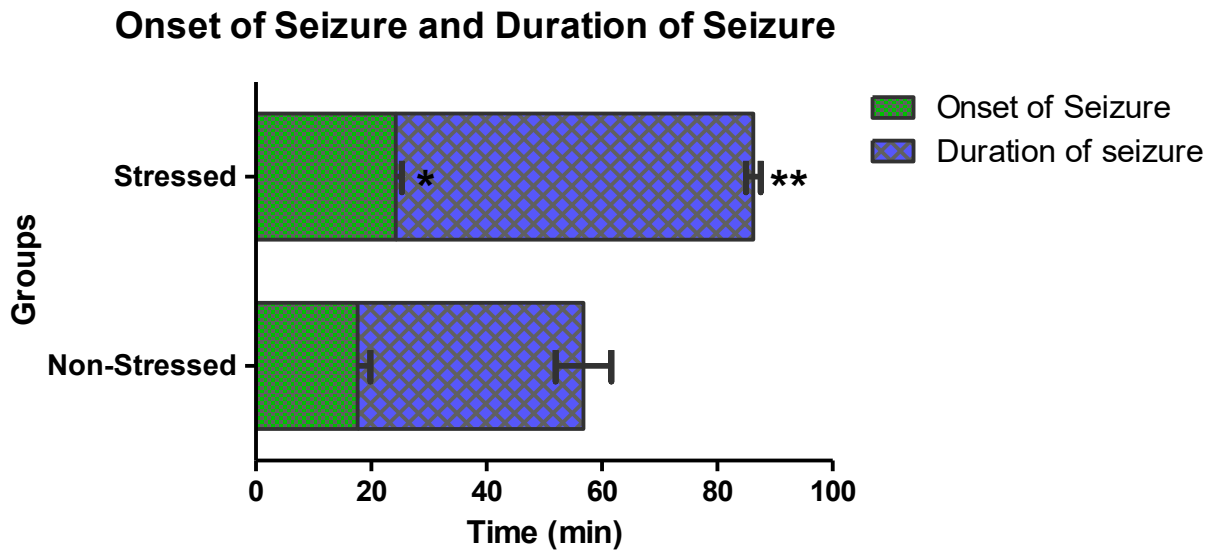

Figure 1. Graph displaying seizure onset and seizure duration in the following groups: non-stressed (NSFS) and stressed (SFS), n=6 per group.*(NSFS onset of seizure vs. SFS onset of seizure) and ** (NSFS duration of seizure vs. SFS duration of seizure), $p < 0.05$.

3.1.2. Elevated Plus Maze: Anxiety-like behaviour

Anxiety-like behaviour was assessed on the following groups of animals on PND 21: non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS). There was a stress effect in both the saline and the febrile seizure groups as these animals spent more time in the closed arms *(NSS vs. SS, $F_{(3.0)} = 97.13$, $p < 0.05$) and *(NSFS vs. SFS, $F_{(3.0)} = 97.13$, $p < 0.05$).

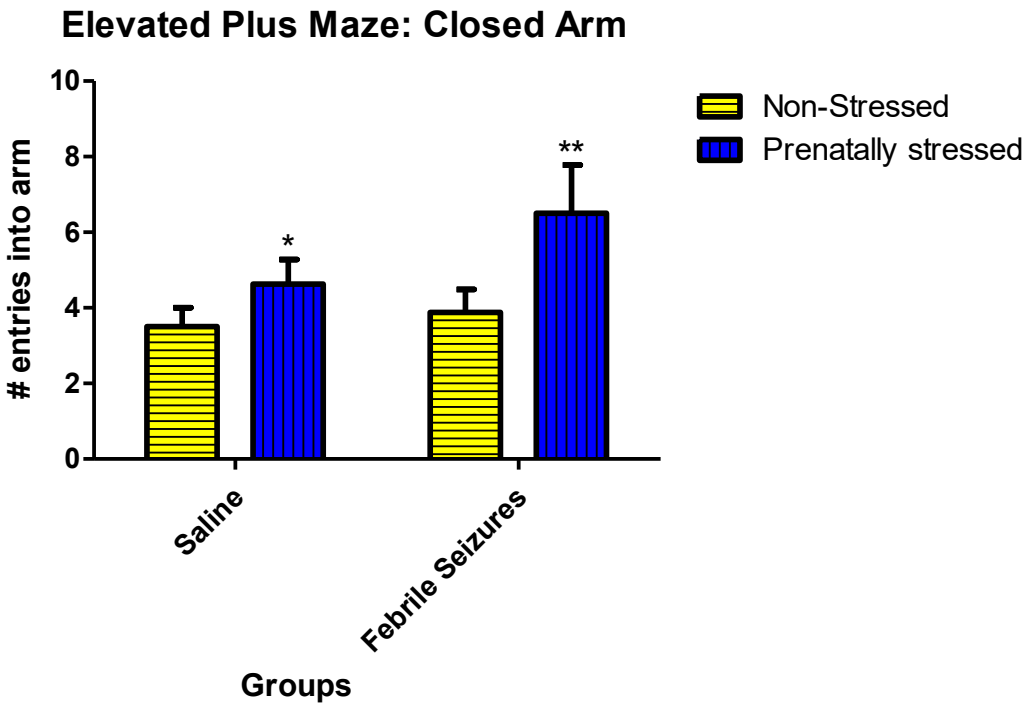

Figure 2. Graph displaying number of entries into the closed arm of the EPM in the following groups: non- stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group.*(NSS vs SS) and **(NSFS vs. SFS), $p < 0.05$.

3.1.3. Brain-Derived-Neurotrophic Factor (BDNF)

To assess a marker of neural plasticity within the hippocampus, BDNF protein concentration was measured in the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS). Animals exposed to prenatal stress showed a stress effect with high concentration of BDNF levels when compared to non-stressed animals *(NSS vs. SS, $F_{(1.0)} = 4.903$, $p < 0.05$)

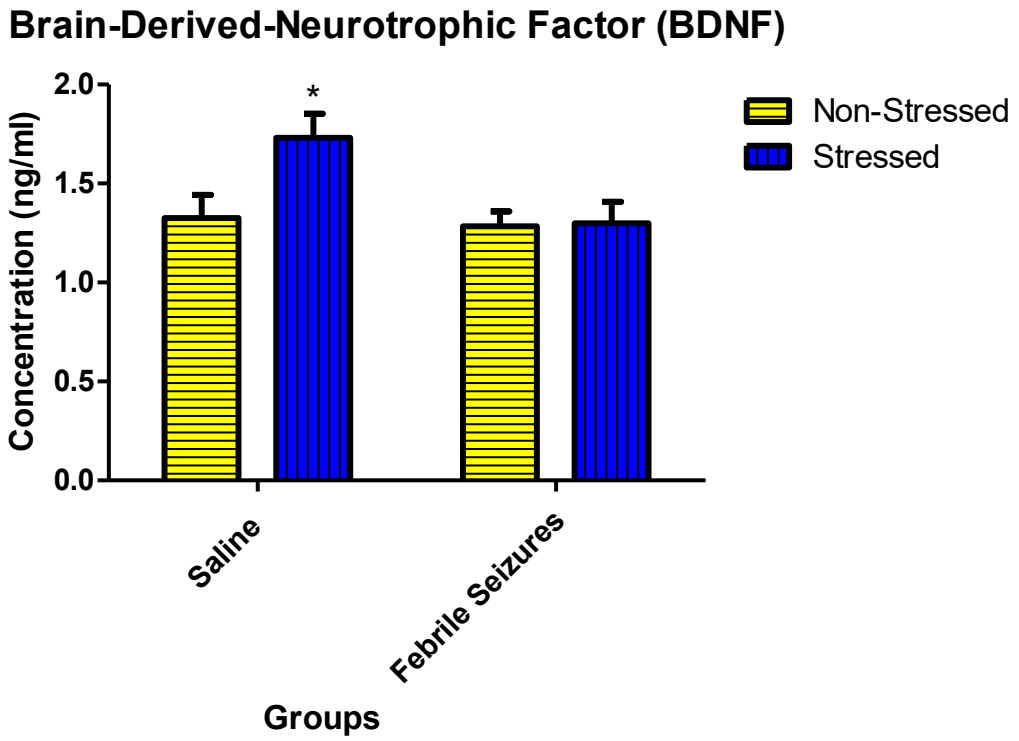

Figure 3. Graph displaying BDNF concentration in the following groups: non- stressed (NSS), non-stressed febrile seizure (NSFS), n=5 per group in duplicate. *(NSS vs SS), $p < 0.05$.

3.1.4. qPCR: REI-1-Silencing Transcription (REST)

The REST factor expression was analysed in the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS) animals.

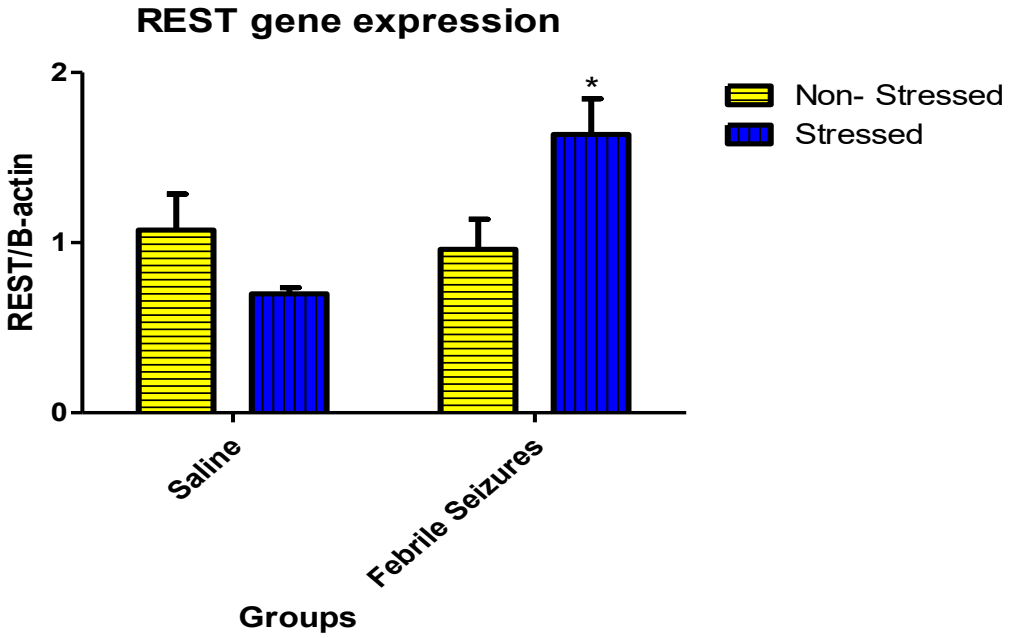

Figure 4. Graph displaying REST gene expression in the following groups: non- stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group.*(NSFS vs. SFS), $p < 0.05$.

There was a stress effect on the REST gene expression in the febrile seizure groups *(NSFS vs. SFS, $F_{(1.0)} = 5.57$, $p < 0.05$).

4.1. Discussion

In our study we investigated the effects of stress and febrile seizure induction on young rats and whether these effects influence the concentration of BDNF and REST gene factor expression in the hippocampus.

Our findings show that animals exposed to prenatal stress had a delay in seizure onset that was followed by a prolonged seizure duration. Stress exerts its effects via various mechanisms (Weinstock, *et al.*, 2008). Exposure to pro-inflammatory cytokine release (IL-1β, TNF-α, IL-6) is a typical response to stress exposure, which increases pro-inflammatory response in the brain resulting in neuronal malfunction and subsequently altered behaviour in rodents (Qulu, *et al.*, 2012; Salim, *et al.*, 2012; Diz-Chaves, *et al.*, 2013). Studies have shown that interleukin-1β results in competitive binding to interleukin-1 receptor type 1, thereby resulting in an

increase in concentration of the neurotransmitter glutamate and an inhibition of the GABAergic system (Viviani, *et al.*, 2003). The subsequent imbalance between the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), leads to excitotoxicity due to inhibition of Ca^{2+} release causing hyperexcitability thereby exaggerating seizure duration and intensity (Viviani, *et al.*, 2003).

Furthermore, our findings showed that exposure to prenatal stress resulted in anxiety-like behaviour in the elevated plus maze (EPM). This confirms a stress effect as stressed animals showed a preference for the closed arms of the EPM. These findings are related to previous studies showing that exposure to prenatal stress results in anxiety-like behaviour (McEwen, 2007; Benoit, *et al.*, 2015). Rodents are nocturnal animals and therefore feel most comfortable in places. It has been shown that in the absence of prenatal stress, rodents are highly explorative in novel environments (Balcombe, 2006). Findings by Weinstock (2008), showed that offspring that were exposed to stress during gestational days 17-21, spent less time in the open arms of the EPM when compared to control animals. In our study, febrile seizure induction did not cause or enhance anxiety-like behaviour in the non-stressed and stressed animals. his suggests that febrile seizures may not have a prolonged effect on HPA axis activity.

Exposure to prenatal stress resulted in an increase in the concentration of BDNF in stressed animals (SS). BDNF is a crucial neurotrophic peptide that has been shown to be involved in many regulatory pathways that contribute to neuronal functioning (Calabrese, *et al.*, 2009; Yoshii & Constantine-Paton, 2009). Exposure to high levels of glucocorticoids during gestation is known to affect the feed-forward mechanism of the HPA-axis which de-sensitizes glucocorticoid receptors in the hippocampus, particularly the dentate gyrus, in brains of rodents (Boyle, 2006; McEwen, 2007; Benoit, *et al*., 2015). This subsequently promotes remodelling of the hippocampus by increasing the levels of extracellular glutamate, resulting in excitotoxic damage and possible cell death via the apoptotic pathway (McEwen, *et al.*, 2012). It is therefore possible that an increase in BDNF concentration at PND21 is an attempt to promote cell survival and regeneration in the presence of the prenatal stress effect. It has been suggested that the presence of high levels of BDNF is an attempt to promote neurogenesis which subsequently dampens the stress effect (McEwen, *et al,* 2015). This has been related to epigenetic and post-translation modifications, as acetylation and methylation have led to changes in BDNF levels resulting in anxiety-like behaviour (McEwen, *et al.*, 2015).

In non-stressed animals, febrile seizures did not affect changes in BDNF concentrations in comparison to the control (NSS) group. These findings suggest that the febrile seizure effect did not result in damage to neurons in these rats. This may be partly due to the fact that febrile seizures are a form of an acute stressor on neuronal networks (Calabrese, *et al.*, 2009) and therefore no permanent changes should be expected as neurotrophic markers aid in the "defensive pathways" to promote neurogenesis (Calabrese, *et al.*, 2009). Thus, in the stressed febrile seizure group of animals, we did not see a rise in the levels of BDNF, as these animals were primarily prenatally stressed. This suggests that prenatal stress is a form of a chronic stress and no neurogenic properties may overcome its detrimental effects.

The REST gene factor expression showed no significant change between non-stressed animals (NSS) and stressed animals (SS). However tendencies of down-regulation in the REST gene factor were seen in the stress group. While this was not a true effect (possibly due to sample size), the REST gene factor down-regulation is usually a sign of neurogenesis (Palm, *et al.*, 1998; Pruunslid, *et al.*, 2011; Cassim, *et al.*, 2015) which supports our BDNF findings.

In the febrile seizure groups a stress effect was present as there was a significant up-regulation in the REST gene factor expression in the stressed animals when compared to non-stressed animals. This suggests that stress in these animals impedes neurogenesis and neuronal plasticity, which is supported by our findings on BDNF concentration results. Palm, *et al.* (1998), showed that the REST gene factor is typically regulated during a seizure as a neuroprotective mechanism, however overexpression of this gene suggests that these animals are susceptible to cognitive impairment in later life. Furthermore, there was no change in the non-stressed febrile seizure exposed animals when compared to the control group, suggesting that our once-off febrile seizure exposure is an acute stressor with only transient effects on neuronal function. These findings reiterate that the stress effect may enhance the febrile seizure effect resulting in a delay in neurogenesis and thus may compromise cognitive function at a later stage.

5.1. Conclusion

Our findings suggest that the prenatal stress effects delay in the onset of seizures and prolongs their duration. Exposure to stress also led to an increase in BDNF concentration (non-seizure group) and an increase in the REST gene expression in rats exposed to febrile seizures. In the face of the non-elevated BDNF concentration, a known neuroprotective and neurogenic

peptide, the increase in the expression of the REST gene may suggest a repression on neural plasticity which may delay cognitive function in the affected animals.

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Disclosure

We hereby disclose that there are no known financial and personal conflict of interests in the work of this project.

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Chapter Four

Article 3

"Methanolic crude extract, *Searsia chirindensis*, attenuates the febrile seizure effect on cognitive function in prenatally stressed adolescent Sprague-Dawley rats"

The manuscript will begin with a title, authors and affiliations on a single page. As this study has been prepared for the journal "**Phytomedicine**" it is written according to the authors guidelines for the journal.

RESEARCH PAPER

The Therapeutic Effects of *Searsia chirindensis*, on Short Term Cognitive Impairment Associated with Febrile Seizure Exposure in Prenatally Stressed Rats.

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Abstract

Drugs used in for the treatment of febrile seizures (FS) may have adverse side-effects on cognitive function in young children. In developing countries traditional beliefs and a lack of financial resources results in many resorting to the use of medicinal plants as an alternative. *Searsia chirindensis* (*Searsia*) is a medicinal plant used in many communities and has been shown to attenuate FS in rats. We investigated the effects of *Searsia* injection on prenatally stressed rats exposed to FS and whether this extract has an effect on cognitive function. Following FS induction and treatment with *Searsia,* the rats were assessed for learning and memory impairment in the Morris-Water maze. Injection of *Searsia* in animals in absence of a seizure impaired learning ability but not in animals exposed to stress and/or FS. No changes were present in memory recall ability in animals. Acetylcholinesterase (AChE) concentrations was increased in prenatally stressed rats but decreased in stressed FS animals. This decrease was attenuated by *Searsia.* The MeCP2 gene expression was up-regulated in non-stressed and *Searsia* treated animals while REST gene expression was down-regulated in non-stressed FS animals with/or without *Searsia* treatment. This suggests that *Searsia* may attenuate transient cognitive impairment following a seizure by activating the cholinergic pathway in the hippocampus and the MeCP2/REST gene expression. Therefore *Searsia* may play a role in the management and attenuation of the short-term cognitive impairment associated with a febrile seizure.

Keywords: Febrile seizures, REST gene, acetylcholinesterase, MeCP2 gene, neuronal plasticity, *Searsia chirindensis*

1.1. Background

A common consequence of compromised neuronal function within the hippocampus is impaired learning and memory (McEwen, 2001; Cassim, *et al.*, 2015). The hippocampus is an area of the brain within the limbic system that is susceptible to insult due to the high expression of mineralocorticoid and glucocorticoid receptors (McEwen, 2001). Neuronal insults resulting from acute stress (once off exposure to a seizure insult) or chronic stress (continuous restraint stress) affect neuronal network functioning (McEwen, 2001). Acetylcholine (ACh) has been shown to influence synaptic neuronal plasticity thereby altering the concentration of neurotransmitters such as glutamate and γ-amino butyric acid (GABA) that determine the fate of cell survival or death in neuronal networks (Picciotto, *et al.*, 2012). Exposure to prenatal stress and febrile seizures has been shown to transiently impair cognitive function in adolescent rats by altering the balance between transcriptional factors methyl-CpG-binding protein 2 (MeCP2) and RE1-silencing-transcriptional (REST) factor (Cassim, *et al.*, 2015).

In humans, febrile seizures are typically remedied with synthetic therapeutic interventions such as carbamazepine, phenobarbital and sodium valporate (Chang, 2014; Lui, *et al.*, 2015). However, after considerable research, studies have shown that the use of these drugs result in adverse side-effects that may lead to mental retardation and the partial loss of cognitive function (Chang, 2014; Lui, *et al.*, 2015). Furthermore, poor economic and health care conditions in developing countries results in many being unable to afford the treatment regimens for convulsion related conditions such as febrile seizures, thus many people resort to the use of traditional remedies which include various plant and herbal extracts.

Searsia chirindensis (*Searsia*), previously called *Rhus chirindensis*, is a plant with many medicinal properties that has been traditionally used by many people in Southern Africa (Ojewole, 2008). *Searsia* also commonly known as Red current, belongs to the Anacardiaceae family that occurs naturally in tropical and sub-tropical regions of Africa, thus making *Searsia* easily accessible to many (Ojewole, 2007; Ojewole, 2008). Various parts of the plant are used for different conditions, for instance, the sap collected from the stem is used to treat cardiovascular complaints, whereas the bark is used to treat rheumatism and mental disorders (Ojewole, 2007). Some of the active ingredients present in the crude extract include flavonoids, which act as antioxidants; saponins that are regarded as anti-carcinogenic, anti-oxidative and anti-diabetic; triterpenes which are regarded as precursors of steroids and possess anti-inflammatory properties as well as, tannins, a polyphenol compound which contributes to

metabolism via its antioxidant properties (Ojewole, 2008). The anti-inflammatory effects of *Searsia* shown by Qulu, *et al.* (2015), suggests that the anti-convulsive effects of *Searsia* may be due to its ability to attenuate the increase in the concentration of pro-inflammatory cytokines such as Interluekin-1β (IL-1β) that have been shown to play a role in febrile seizure occurrence.

In this study we investigated whether *Searsia* crude bark methanolic extract injection attenuates the transient cognitive impairment that follows febrile seizure induction and whether MeCP2/REST gene expression, as well as acetylcholine concentration play a role in this effect.

2.1. Materials & Methods

2.1.1. Animals

Male and female Sprague-Dawley rats were obtained from the Biomedical Resource Centre (BRC) of the University of KwaZulu-Natal. The animals were housed under standard BRC conditions (temperature of ±23˚C and 20% humidity) and a 12 hour light/dark cycle (lights on at 06h00) was maintained. Food and water were available *ad libitium*. All experimental procedures were approved by the Animal Ethics Research Committee of the University of KwaZulu-Natal (Ethical Number: 73/14/animal) and were in accordance with guidelines of the National Institute of Health, USA.

2.1.2. Prenatal Handling

Mating

Ten female Sprague-Dawley rats were housed in pairs for a week so as to synchronise their oestrus cycles. Vaginal smears were taken daily so as to identify when the rat's cycle was in pro-oestrus At pro-oestrus, a male rat was placed in the cage and the following morning, the presence of sperm positive cells in the vaginal smear was regarded as an indicator of successful mating (Shorr, 1941; Nelson *et al*., 1982; Hubscher *et al.*, 2005; Moosa, *et al.,* 2013). This was regarded as gestational day (GND) 1. Following a successful mating, the male rat was removed from the cage.

Prenatal Stress Protocol

The prenatal stress protocol was followed as per Cassim, *et al.* (2015). Briefly, on GND 14, pregnant dams were divided into 2 groups: a non-stressed and a stressed group. The stressed rats were taken daily to a separate room and placed in rodent restrainers for 1 hour, for 7 days

starting at 09h00. The restrained rats were returned to their home room at the end of every stress period protocol.

2.1.3. Post-Natal Handling

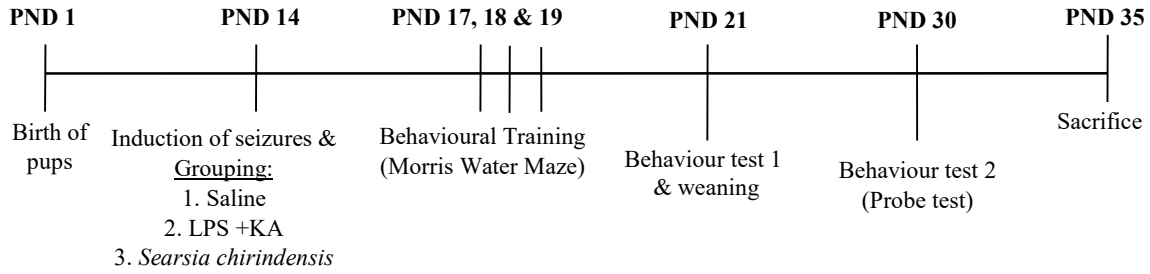

Figure 1. Linear flow diagram depicting the post-natal handling protocol

Following birth, the pups remained with their dams undisturbed until PND 14 after which they were divided into the following groups (n=6 per group);

Non-stressed animals

- Non-stressed animals injected with saline (NSS)
- Non-stressed with injected with lipopolysaccharide (LPS) and kainic acid to induce a febrile seizure (NSFS)
- Non-stressed animals injected with *Searsia* (NS-S)
- Non-stressed animals injected with lipopolysaccharide and kainic acid to a febrile seizure and then treated with *Searsia* (NSFS-S)

Prenatally stressed animals

- Stressed animals injected with saline (SS)
- Prenatally stressed animals injected with lipopolysaccharide and kainic acid to induce a febrile seizure (SFS)
- Stressed animals injected with *Searsia* (S-S)
- Stressed animals injected with lipopolysaccharide and kainic acid to induce a febrile seizure and then treated with *Searsia* (SFS-S)

The experimental protocol was performed as described by Cassim, *et al.* (2015). Briefly, control animals (NSS and SS) were injected with 0.2 ml/g saline (0.9% NaCl, Adcock Ingram, South Africa). To induce a febrile seizure on postnatal day (PND) 14, NSFS, SFS, NSFS-S and SFS-S rats were injected with 0.2 ml/kg of LPS (200 µg/kg, Sigma, USA). Following injection, the pups were returned to their dams to allow for suckling and grooming as so to avoid dehydration and hypothermia. Two and a half hours later, the pups were once again removed from their dams and injected with 0.2 ml/g kainic acid (1.75 mg/kg, i.p, Sigma, USA) to induce a febrile convulsion (Heida, *et al*., 2004; Qulu, *et al.*, 2012). Once the pups had reached stage 3 of seizures i.e. myoclonic jerks throughout the body of the rat (Table 1) (Heida, *et al.*, 2004; Ojewole, 2008), 0.2 ml/g of the *Searsia chirindensis* crude bark extract working solution (1000 mg/kg, i.p.) was administered. Following *Searsia* injection, convulsive behaviour of each rat was monitored and recorded for a period of 60 minutes. The various stages of convulsive behaviour were assessed following the guidelines provided in Heida, *et al.* (2004) and Ojewole (2008) (Table 1). Thereafter, pups were returned to their dams.

Table 2. Stages involved in the progression of a febrile seizure (Heida, *et al.*, 2004; Ojewole, 2008)

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

2.2.1. *Searsia chirindensis*

a. Plant material

Fresh stem-bark pieces of *Searsia chirindensis* were collected from University of KwaZulu-Natal's Howard College Campus between May and August. The stem-bark was identified by a botanist from the University's Botany Department as that of *Searsia chirindensis* (family:

Anacardiaceous). A voucher specimen of the plant (voucher number: 4594000, assertion no. 1228/ward herbarium) was deposited in the University's Botany Departmental Herbarium.

b. Preparation of *Searsia chirindensis* stem-bark methanol extract

Fresh pieces of the stem-bark were air-dried at room temperature. One kilogram (1 kg) of the air-dried *Searsia chirindensis* stem-bark pieces were pounded and milled into fine powder using a commercial grinder. The stem-bark powder was extracted once, with 0.5 litres of methanol (Sigma, USA) to 250g of *Searsia c.* powder using filter paper over a period of 48 hours (with occasional shaking and tampering) at room temperature (23±1 ◦C). This resulted in a yield of 250g of thick viscous extract which was freeze-dried under reduced pressure in a rotary evaporator at 60±1 ◦C (Cole-Parmer, South Africa). Following freeze-drying we ended up with a 30g yield of a red brown-copper, powdery, crude *Searsia chirindensis* stem-bark extract. Without any further purification, the crude extract was used in this study.

On the day the rats were injected, aliquoted portions per animal (1000mg/kg) were dissolved in 15ml of saline working solution (0.9% NaCl, Adcock Ingram, South Africa) and injected into our rats.

2.2.2. Behavioural Tests

Morris Water Maze

This test assesses the learning and recall ability of rats which covers exploratory, navigational, spatial and contextual memory (Morris, 1984; D'Hooge & De Deyn, 2001; Garthe & Kempermann, 2013).

Training in the Morris Water Maze (MWM) took place between PND 17-19. This was followed by two test phases on PND 21 and PND 30 which assessed learning and memory deficits respectively.

Training and testing procedures

Animals were subjected to three training sessions over a period of three days (PND 17-19). Animals were taken to the behavioural room one hour prior to training to allow for acclimatisation to the new environment. For the training procedure and tests, the protocol followed was described in Cassim, *et al.,* 2015. Briefly, 21 day old rats were placed in a quadrant other than the hidden platform and the time to locate the hidden platform was recorded. Animals were then returned to their cages before being returned to their room. Animals were left undisturbed from PND 22-29. The probe test was conducted on PND 30. The animals underwent the same acclimatisation procedure previously described prior to the probe test. For the probe test, the platform was removed completely from the maze and the animals were allowed 120 seconds in the water. Time spent in the quadrant of the hidden platform was timed. Following the probe test, the animals were returned to their home room. Animals were sacrificed by decapitation 5 days later (PND 35), three weeks after seizure induction.

2.2.3. Sacrifice

Rats were sacrificed by decapitation on PND 35. Hippocampal tissue was collected and weighed prior to freezing in liquid nitrogen. All tissue material was stored in a bio-freezer at -80 ˚C.

2.2.4. Neurochemical Analysis

a. Real-Time PCR (qPCR)

The qPCR analysis was done as previously described (Cassim, *et al.*, 2015). Briefly, hippocampal tissue (50mg) was homogenized and suspended in 400µl of lysis buffer (Zymo Research, USA). This was followed by RNA extraction using the guidelines stipulated by the manufacturer (ZR RNA MiniPrep™, USA). Purification of RNA isolates was conducted using a NanoDrop. Purified RNA isolates were used in conjunction with the iScript™ cDNA Synthesis Kit (Biorad, South Africa), to construct cDNA using a Thermocycler as per conditions stipulated by the manufacturer. The Fast start SYBR green kit (Roche Diagnostics,

USA), cDNA and primers were used to amplify our gene of interest at optimised conditions in the Lightcycler 480 (Roche Diagnostics, USA).

The following primer sequences were used:

Primer sequences:

- MeCP2 gene- Forward primer: CGTCCCCTTGCCTGAAGGTTGGA,
  Reverse primer: CTTTCCAGCAGAGCGACCAG;
- REST gene- Forward primer: CAGTTGAACTGCCGTGGG,
  Reverse primer: CATCCGCTGTGACCGCTG;
- Beta actin gene- Forward primer: GCTTCTTTGCAGCTCCTTCGT,
  Reverse primer: CCAGCGCAGCGATATCG

(Inqaba, South Africa).

b. Immunoassay (ELISA)

Acetylcholineesterase (AChE) was measured with a conventional enzyme-linked immunosorbent assay (ELISA) system. The AChE immunoassay (ELabSciences, WuHan, China) was performed according to the manufacturer's protocol. The harvested hippocampal tissue was rinsed and incubated in 1 ml of PBS overnight at -20°C. After two freeze thaw cycles, homogenates were centrifuged at 5000xg at 7°C using a Heraeus Labofuge 200 (USA). The supernatant was used to determine the AChE concentration according to the protocol provided by the manufacturer (ELab. The sensitivity of the assay was 0.78 ng/ml-50 ng/ml, and the cross-reactivity was minimum. The intra- and inter-assay precisions were <10%, respectively.

2.2.5. Statistical Analysis

The data was analyzed using the software GraphPad Prism (version 5). Normality and Gaussian distribution were determined using the Kolmogorov-Smirnov test. A two-way factorial analysis of variance was performed with stress and febrile seizures, stress and *Searsia* and febrile seizures and *Searsia* as between rat factors. Significant main effects were followed by the *Bonferonni* post hoc test. $p < 0.05$ was considered significant.

3.1 Results

3.1.1. Morris Water Maze

The following 8 groups were assessed for learning deficits on PND 21; non-stressed saline (NSS), stressed saline (SS), non-stressed with exposure to febrile seizures (NSFS), stressed with exposure to febrile seizures (SFS), non-stressed with *Searsia* only (NSS-S), stressed with *Searsia* only (SS-S), non-stressed with exposure to febrile seizures (NSFS-S) and *Searsia* and stressed with exposure to febrile seizures and *Searsia* (SFS-S). A *Searsia* effect was present in the non-stressed (NS) groups as these rats took a longer time to locate the hidden platform *(NSS vs. NS-S, $F_{(1.0)} = 5.33$, $p < 0.05$).

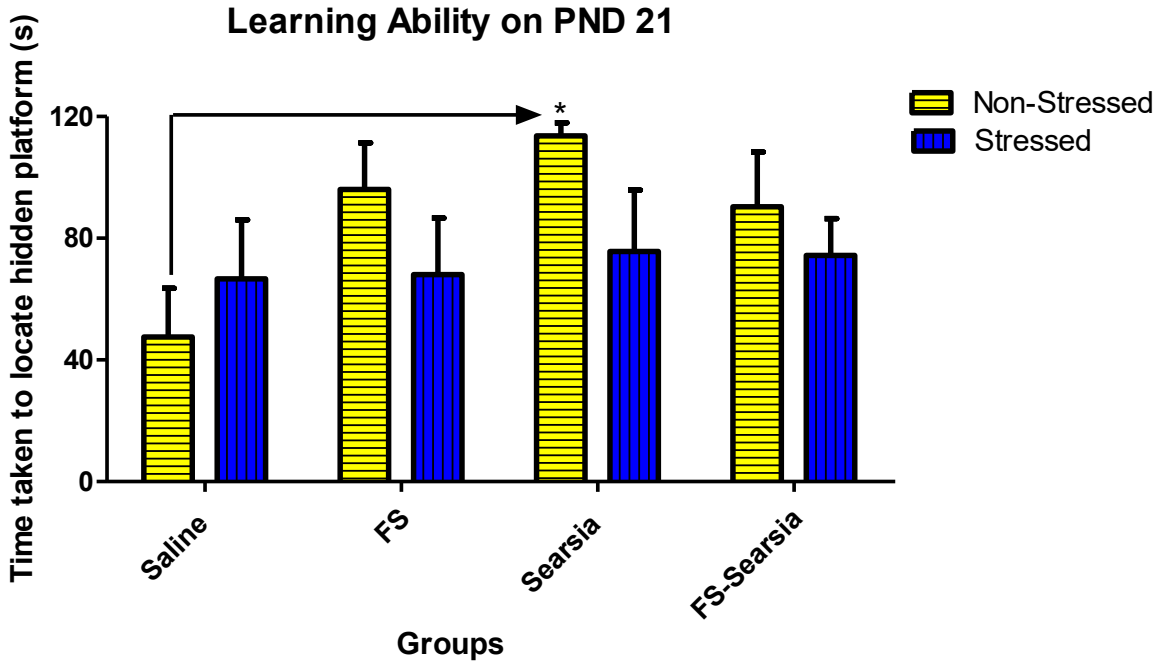

Figure 3.1**.** Graph displaying average time taken to locate the hidden platform in the MWM in the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with exposure to febrile seizures (NSFS), stressed with exposure to febrile seizures (SFS), non-stressed with *Searsia* only (NS-S), stressed with *Searsia* only (S-S), non-stressed with exposure to febrile seizures (NSFS-S) and *Searsia* and stressed with exposure to febrile seizures and *Searsia* (SFS-S), n=6 per group.*(NSS vs. NS-S), $p < 0.05$.

Just over a week later (PND 30), the groups were assessed for memory deficits using the probe tests. There were no significant changes observed in all groups.

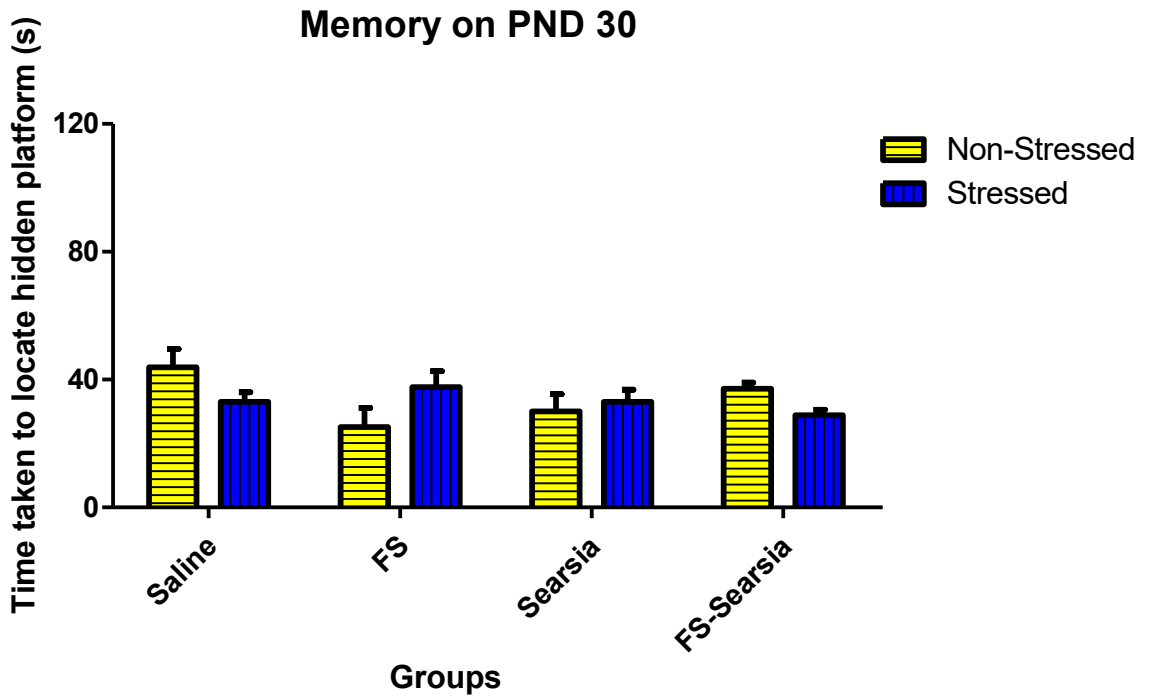

Figure 3.2. Graph displaying average time spent in the quadrant with hidden platform in the MWM in the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with exposure to febrile seizures (NSFS), stressed with exposure to febrile seizures (SFS), non-stressed with *Searsia* only (NS-S), stressed with *Searsia* only (S-S), non-stressed with exposure to febrile seizures (NSFS-S) and *Searsia* and stressed with exposure to febrile seizures and *Searsia* (SFS-S), n=6 per group, $p < 0.05$.

3.1.2. Acetylcholinesterase (AChE) Enzyme

Hippocampal AChE concentration was measured in the 8 groups previously described. A stress effect was present in all groups with the exception of the groups receiving *Searsia* only *(NSS vs SS, $F_{(1.0)} = 94.98$ $p < 0.05$); **(NSFS vs SFS, $F_{(1.0)} = 94.98$, $p < 0.05$) and ***(NSFS-S vs SFS-S, $F_{(1.0)} = 94.98$, $p < 0.05$ $F_{(1.0)} = 94.98$, $p < 0.0$). A *Searsia* effect was present in the non-stressed group [1](NSS vs NS-S, $F_{(1.0)} = 64.62$, $p < 0.05$).

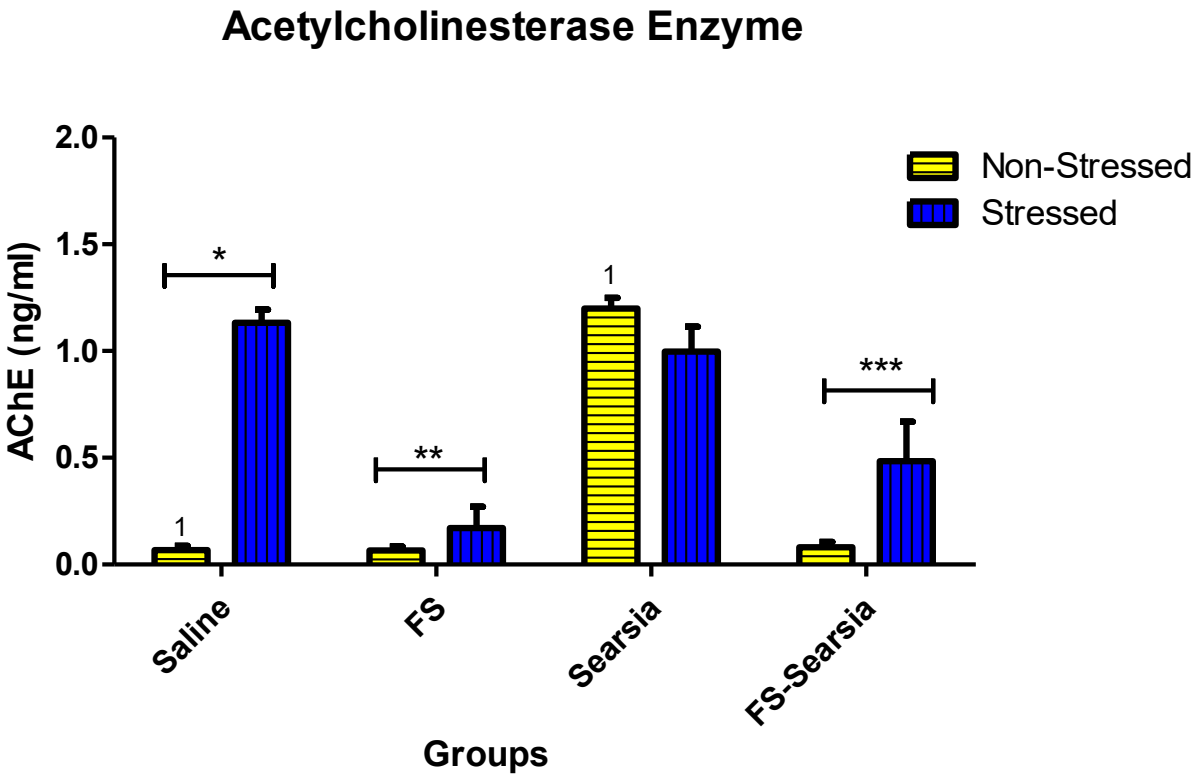

Figure 3.3. Graph displaying hippocampal AChE Enzyme concentration on PND 35 the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with exposure to febrile seizures (NSFS), stressed with exposure to febrile seizures (SFS), non-stressed with *Searsia* only (NS-S), stressed with *Searsia* only (S-S), non-stressed with exposure to febrile seizures (NSFS-S) and *Searsia* and stressed with exposure to febrile seizures and *Searsia* (SFS-S), n=6 per group.*(NSS vs. SS), **(NSFS vs. SFS), ***(NSFS-S vs. SFS-S), [1](NSS vs NS-S), $p < 0.05$).

3.1.3. RT-PCR: MeCP2 and REST genes

MeCP2 gene expression was analysed in the 8 groups described above. A stress effect was present in all groups except the group of animals exposed to febrile seizures *(NSS vs. SS, $F_{(1.0)} = 6.80$, $p < 0.05$); **(NS-S vs. S-S, $F_{(1.0)} = 6.80$, $p < 0.05$) and ***(NSFS-S vs. SFS-S, $F_{(1.0)} = 13.39$, $p < 0.05$). A *Searsia* effect was present in the non-stressed febrile seizure rats and the non-stressed febrile seizure with *Searsia* exposure rats [1](NSFS vs. NSFS-S, $F_{(1.0)} = 18.32$, $p < 0.05$).

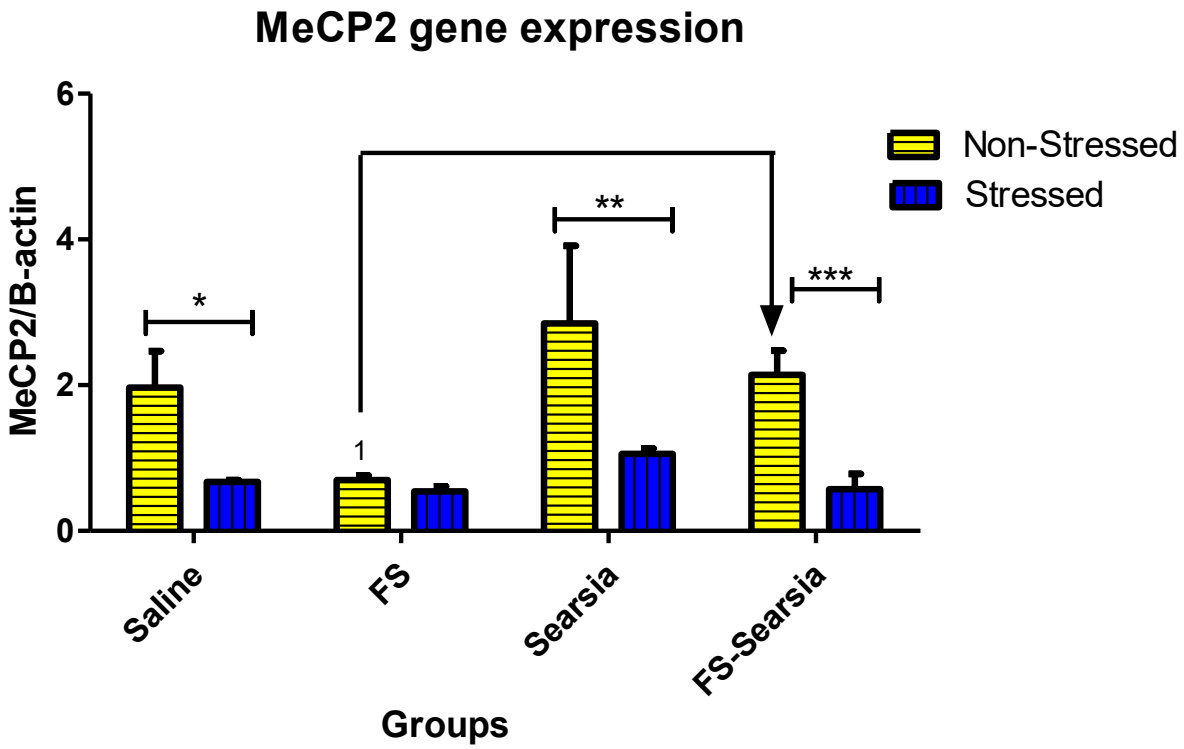

Figure 3.4. Graph displaying the MeCP2 gene expression on PND 35 the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with exposure to febrile seizures (NSFS), stressed with exposure to febrile seizures (SFS), non-stressed with *Searsia* only (NS-S), stressed with *Searsia* only (S-S), non-stressed with exposure to febrile seizures (NSFS-S) and *Searsia* and stressed with exposure to febrile seizures and *Searsia* (SFS-S), n=6 per group..*(NSS vs. SS), ** (NS-S vs. S-S), *** (NSFS-S vs SFS-S) and [1](NSFS vs NSFS-S), $p < 0.05$).

The REST gene expression was analysed in the same groups as the MeCP2 gene expression. A febrile seizure effect was present in the non-stressed groups *(NSS vs. NSFS, $F_{(1.0)} = 5.39$, $p < 0.05$) and **(NS-S vs. NSFS-S, $F_{(1.0)} = 5.39$, $p < 0.05$). A *Searsia* effect was present in the non-stressed and prenatally stressed animals groups exposed to febrile seizures ***(NSFS vs. NSFS-S, $F_{(1.0)} = 5.39$, $p < 0.05$) and [1](SFS vs. SFS-S, $F_{(1.0)} = 5.39$, $p < 0.05$).

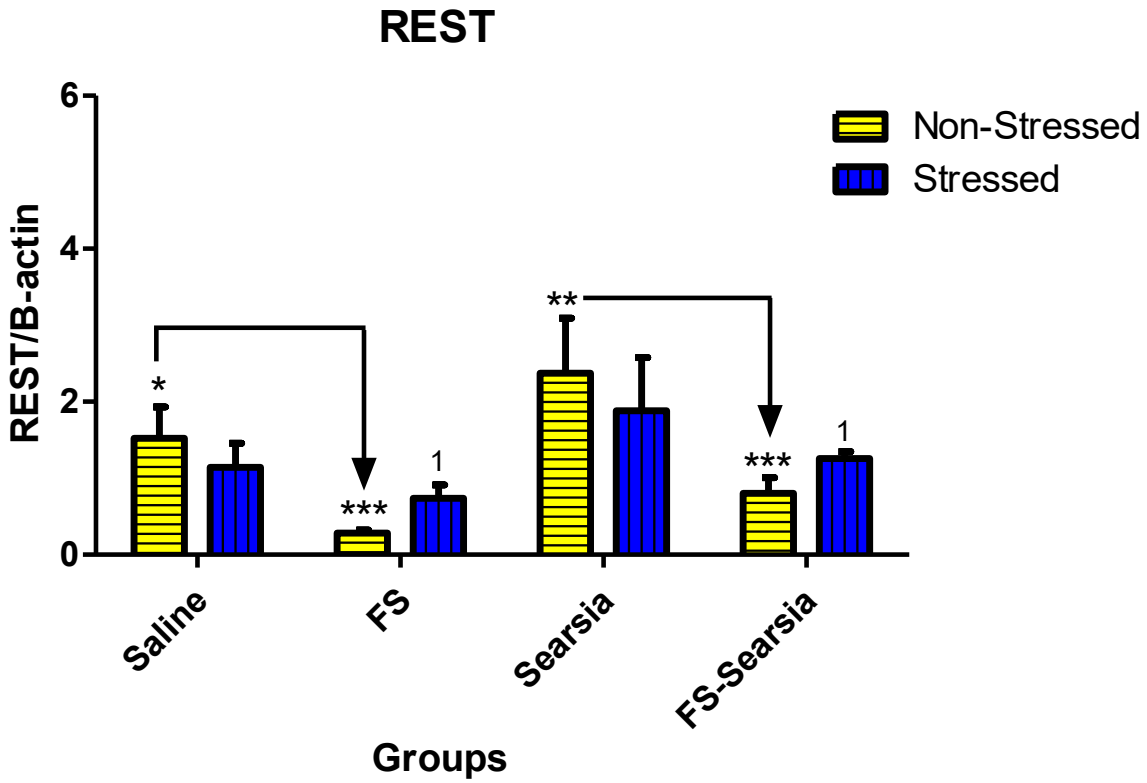

Figure 3.5. Graph displaying the REST gene expression on PND 35 the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with exposure to febrile seizures (NSFS), stressed with exposure to febrile seizures (SFS), non-stressed with *Searsia* only (NS-S), stressed with *Searsia* only (S-S), non-stressed with exposure to febrile seizures (NSFS-S) and *Searsia* and stressed with exposure to febrile seizures and *Searsia* (SFS-S), n=6 per group..*(NSS vs. NSFS), **(NS-S vs. NSFS-S), ***(NSFS vs NSFS-S) and [1](SFS vs SFS-S), $p < 0.05$).

4.1. Discussion

In our study we investigated the effects of the *Searsia* crude bark extract on learning and memory function in prenatally stressed febrile seizure exposed rats. We further investigated whether *Searsia* treatment affected the concentration of the acetylcholinesterase enzyme and the expression of MeCP2 and REST genes in the hippocampus.

Assessment in the Morris water maze (MWM) showed that injecting *Searsia* in the absence of a febrile seizure in non-stressed animals resulted in poor learning ability. This suggests that injecting *Searsia* in the absence of convulsions may impair the neuronal plasticity needed for learning new tasks. While there was a tendency for prenatally stressed animals and febrile seizure exposed rats to take longer to locate the hidden platform, this effect was not severe as in the non-stressed rats treated with *Searsia.* Treatment with *Searsia* in rats exposed to febrile seizures did not affect the ability to learn. Previously, we had shown that *Searsia* treatment during a seizure dampens seizure severity and attenuates Interleukin-1β (IL-1β) concentration

(Qulu, *et al.*, 2015). Therefore the improved learning ability in the febrile seizure exposed rats treated with *Searsia* may suggest a protective effect against neuroinflammation and potential apoptotic cell death.

Just over a week after the learning assessments were performed, these animals were subjected to the probe test to assess memory recall ability. As suggested by the time period the animals spent in the quadrant with the hidden platform, no lingering effects of exposure to febrile seizures and *Searsia* were observed. This suggests that the changes and tendencies seen at PND 21 were transient and that treatment with *Searsia* does not have chronic effects on hippocampal neuronal plasticity. We suggest that the effects on learning seen in the non-stressed rats injected with *Searsia* only (NS-S) may be due to the crude extract's anti-inflammatory properties which may cause inhibition in the release of pro-inflammatory cytokines involved with memory formation. Tumor necrosis factor-α (TNF-α) and IL-1β are cytokines that are produced in the brain under normal conditions and they are regarded pivotal for normal synaptic functioning (Marin & Kipnis, 2013; Vezzani, *et al.,* 2016). TNF-α acts on the TNFR2 receptor to promote neurogenesis (Iosif, *et al.*, 2006) while the IL-1 receptor contributes to learning tasks in the Morris water maze (Goshen, *et al.*, 2007). These studies concluded that impairment or reduction in these signalling pathways resulted in cognitive deficits (Iosif, *et al.*, 2006; Goshen, *et al.*, 2007). As previously discussed, *Searsia* has anti-inflammatory properties and has been shown to directly influence the concentration of IL-1β (Qulu, *et al.*, 2015).

The acetylcholinesterase (AChE) enzyme is responsible for the degradation of the neurotransmitter, Acetylcholine (ACh) that influences synaptic transmission and induces synaptic plasticity in neurons (Picciotto, *et al.*, 2012). We showed that exposure to prenatal stress increased hippocampal AChE concentration. This suggests an increase in ACh degradation in the synaptic cleft. It has been shown that an increase in ACh degradation results in decreased plasticity following exposure to prenatal stress (Picciotto, *et al.*, 2012).

Furthermore, we showed that a febrile seizure effect (decrease in AChE concentration) in non-stressed animals treated with *Searsia* was present, however treatment in stressed animals resulted in an increase in the AChE concentration. This suggests that ACh degradation was reduced in the non-stressed animals while it was increased in stressed animals. We therefore infer that *Searsia* treatment in non-stressed animals promoted neuronal plasticity while in the stressed animals it did not attenuate the stress effect on cognitive function (less neuronal plasticity). ACh signalling is regulated by chloride transporters (Picciotto, *et al.*, 2012). These

chloride transporters are important as their effects of GABA bidding to a cell results in neuronal hyperpolarisation decreasing calcium ion entry into the cell (Picciotto, *et al.*, 2012; Granger, *et al.*, 2015). Hyperpolarisation of GABA subsequently inhibits neurons by reducing electrical activity (Picciotto, *et al.*, 2012; Granger, *et al.*, 2015), therefore treatment with *Searsia* resulted in an increase in ACh concentration (decrease AChE concentration) in the pre-synaptic cleft thus attenuating febrile seizure convulsions as seen in Qulu, *et al.* (2015). This suggests that ACh has a dual role in seizure pathology i.e. by increasing ACh concentration (decreasing AChE concentration) it plays an important function in seizure attenuation which may explain the shorter seizure duration in non-stressed animals as described in Qulu, *et al.* (2015).

Hippocampal MeCP2 gene expression showed down-regulation in the expression of MeCP2 in the stressed rats except in the untreated febrile seizure groups. As MeCP2 functions to promote neurogenesis and neuronal plasticity, this suggests that these functions are slowed by exposure to stress. This supports studies that have shown that exposure to early life stress such as prenatal stress may result in the development of cognitive deficits (McEwen, 2000; McEwen, *et al.*, 2012; Qulu, *et al.*, 2012; Cassim, *et al*., 2015).In the absence of treatment, both non-stressed and stressed febrile seizure groups exhibited down-regulation in the expression of the MeCP2 gene. This may suggest that while the febrile seizure effect is transient, it however does slow down MeCP2 related functions on neuronal plasticity. Treatment with *Searsia* in non-stressed febrile seizure exposed rats resulted in the up-regulation of MeCP2 gene expression. Previously we showed that exposure to febrile seizures down-regulated MeCP2 gene expression in the hippocampus (Cassim, *et al.* 2015), hence an up-regulation of MeCP2 in these animals following injection with *Searsia* suggests that in addition to attenuating seizure development, *Searsia* also promotes neuronal plasticity in vulnerable neurons. In our study, *Searsia* was not able to reverse the deleterious effects of stress on the hippocampal neuronal circuitry nor plasticity, as these effects occurred *in utero,* weeks before the injection with *Searsia.*

In the non-stressed rats exposed to febrile seizures (untreated and treated) the REST gene expression was down-regulated, suggesting that in these animals neuronal plasticity is being favoured. These findings reiterate that prenatal stress has detrimental effects on neuronal plasticity whereas febrile seizures transiently affects neuronal function however, exposure to *Searsia* crude bark extract attenuates these effects by promoting potentiating therapeutic effects on neuronal plasticity.

5.1 Conclusion

The outcomes of our findings suggest that *Searsia* is able to attenuate febrile seizures by influencing the release of AChE. Exposure to prenatal stress compromises neuronal function and plasticity, subsequently affecting the animals' ability to learn. This effect is also present in following exposure to febrile seizures but is transient in these rats and may be influenced by the concentration of cytokines. In the presence of febrile seizures, *Searsia* injection seems to counteract the impaired ability to learn. This effect together with its ability to attenuate seizure progression suggests that *Searsia* may have potential for use as an adjunct treatment for febrile seizures.

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Disclosure

We hereby disclose that there are no known financial and personal conflict of interests in the work of this project.

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Chapter Five

Conclusion

Febrile seizures results from a common infection that evokes a fever that subsequently triggers the firing of neurons resulting in a seizure. This condition is exacerbated by exposure to stress during pregnancy. In these prenatally stressed rats, seizures were more vigorous and took longer to abate than in non-stressed rats. Exposure to stress and febrile seizures also affected the ability to learn and remember in the Morris water maze. However, these cognitive deficits were transient as the gene expression results suggested that there may be an attempt to promote neural plasticity following the febrile seizure episode. We also showed that exposure to stress early in life may affect the ability to promote neural plasticity in the hippocampus as evidenced by the increase in the MeCP2 gene expression and corresponding decrease in the expression of the REST gene. These genes work together in promoting neuronal plasticity (and hence learning and memory) with an increase in the MeCP2 gene expression promoting plasticity.. Exposure to stress also led to an increase in BDNF concentration (non-seizure group) and an increase in the REST gene expression in rats exposed to febrile seizures. In the face of the non-elevated BDNF concentration, a known neuroprotective and neurogenic peptide, the increase in the expression of the REST gene may suggest a repression on neural plasticity which may delay cognitive function in the affected animals. Treatment with *Searsia chirindensis* attenuated febrile seizures by influencing the release of AChE and increasing the expression of the MeCP2 gene. Exposure to prenatal stress compromises neuronal function and plasticity, subsequently affecting the animals' ability to learn. In the presence of febrile seizures, *Searsia* injection seems to counteract the impaired ability to learn. This effect together with its ability to attenuate seizure progression suggests that *Searsia* may have potential as an adjunct treatment for febrile seizures.

In conclusion our study confirms that febrile seizures remain a benign condition but immediate following of a seizure episode, there seems to be a transient impairment in cognitive function related to learning and memory function. While treatment with *Searsia* is able to attenuate seizure development in all animals, it however does not seem to reverse neuronal malformation present in prenatally stressed animals. We therefore conclude that the crude bark extract of *Searsia chirindensis* contains bioactive ingredients that may be used as an adjunct treatment for the management of febrile seizures.

Chapter Six

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Addendum A

Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats.

Addendum B

Febrile seizure induction impairs neuronal plasticity in prenatally stressed rat offspring.

Addendum C

Protocol of qPCR

1. Gene Expression studies

There are three important steps in this part of the study:

1. RNA isolation
2. cDNA Synthesis
3. Real-time RT PCR

1.1 RNA Isolation

RNA was isolated, from the tissues using the RNeasy Mini Kit. The kit contains the following reagents and apparatus:

- Lysis buffer
- RNeasy Spin Columns
- Buffer (Prep)
- Buffer (Wash)
- RNase Free Water

In addition to these materials supplied in the kit the following is also needed:

- 96 – 100% Ethanol
- 70% Ethanol
- Collection Tubes (1.5 and 2.0 ml)

The procedure followed is explained below:

1. The tissues that were stored in the Bio-freezer at -75°C were removed and approximately 40 to 50 mg of tissue was weighed out.
2. The tissue was placed in a 2.0ml Eppendorf tube with 700 µl of lysis reagent and then disrupted using a Sonicator (important to note that this was done on ice to maintain the integrity of the RNA).
3. The tubes of tissue were centrifuged at 12 000 rpm for 15 min at 4°C, after which the centrifuged was heated to room temperature (15 – 25°C).
4. 600 µl of the upper aqueous phase was transferred to a new micro centrifuge tube.
5. An equal volume (600 µl) of ethanol was added to the tube, no centrifugation or mixing was done at this step.
6. 700 µl of the sample was then added to an Rneasy Mini spin column that is placed in a 2 ml collection tube (was supplied in the kit) and was then centrifuged for 15s at 10 000 rpm at room temperature. The flow-through was discarded.
7. 700 µl of Buffer (Wash) was added to the spin column and spun at 10 00 rpm for 15s, the flow-through was discarded this step was done to wash the membrane.
8. 500 µl of Buffer (Prep) was added to the spin column and spun at 10 000 rpm for 15s, the flow-through was discarded.
9. Step 12 was repeated only increasing the centrifugation time to 2 min.
10. 30 µ of RNase-Free water was added to elute the RNA and was spun at 10 000 rpm for 1, after centrifuging a second volume (30 µl) of RNase-Free water was added and spun down again the RNA was then collected from the collection tube and stored in the Bio-Freezer until it was time for cDNA synthesis.

1.2 Assay to determine the Purity and Concentration of RNA

RNA concentration and purity was determined using the NanoDrop ND- 1000 Spectrophotometer. The spectrophotometer was blanked using 2 µl of distilled water, 2 µl of the RNA sample was then placed on the pedestal absorbance was read at 230 nm, 260 nm and 280 nm. The ratio of the absorbance at 260 nm and 280 nm ($\frac{A\ 260\ nm}{A\ 280\ nm}$) as this is an indication of the purity of RNA, samples with values between 1.7 – 2.1 are considered to contain RNA of good purity. The software that accompanies the NanoDrop is able to calculate the concentration of RNA in ng/µl.

1.3 cDNA Synthesis

Reverse transcriptase PCR was used to synthesize cDNA from the RNA isolated in the previous step. This was done using the BIORAD iScript cDNA Synthesis Kit. The kit contains all the reagents needed for the reaction process and is shown in the table below:

Table 1.3.1: showing contents of cDNA synthesis kit and volumes required for Reaction mixture

Reagent	Volume (µl)
Nuclease Free Water	8
5x iScript Reaction mix	4
iScript Reverse Transcriptase	1
RNA Template (isolated in 3.4.2)	7
Total Volume	20

The reagents mentioned above were added to a thin-walled PCR tube and incubated in the Applied Biosystems GeneAmp 9700 thermocycler under the following conditions:

- 5 minutes at 25 °C
- 30 minutes at 42 °C
- 5 minutes 85 °C
- A final Hold at 4 °C

The tubes were then stored in Bio-freezer at – 75 °C to be used in the Real-time PCR detection of our desired genes.

1.4 Real -Time PCR Detection

The real time detection was done using the Roche Diagnostics Lightcycler. The Fast Start SYBR green kit was used, the kit also from Roche Diagnostics and contains the reagents for the PCR reaction including nuclease free water, $MgCl_2$ and SYBR green (the fluorescent dye) we had to order our own primers and use our own cDNA that was synthesized in 3.4.3.

In order to conduct the reaction a number of reagents need to be added to the reaction; this mixture of reagents is called the master mix and is prepared according to the table below:

Table 1.4.2: showing reagents and volumes used in the preparation of the Master Mix

Reagent	Volume (µl)
Nuclease Free Water	5.5
$MgCl_2$	2.0

Forward Primer	0.25
Reverse Primer	0.25
Fast Start SYBR Green I	1.0
cDNA (made in 3.4.3)	1.0

The cDNA for each of the control group were pooled together and run in the Lightcylcer. A standard curve was constructed using the cDNA from the control groups and dilutions were done according the table shown below:

Table 1.4.3: showing Serial Dilutions for Standard Curve

Dilution Series	**Volume of cDNA (µl)**	**Volume of Nuclease Free Water (µl)**	**Concentration (U)**
S_1	2 µl from each control	0	1000
S_2	4 µl of S_1	16	200
S_3	2 µl of S_1	18	100
S_4	4 µl of S_3	16	20
S_5	2 µl of S_3	18	10
S_6	4 µl of S_5	16	2
S_7	2 µl of S_5	18	1

Addendum D

Protocol of BDNF ELISA

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Addendum E

Protocol of AChE ELISA

Addendum F

Conference Output

1. International Society of Neuroscientists in Africa (SONA) (year 2015)

   Oral presentation- Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats.

2. University of Pretoria Neuroscience Day (year 2015)

   Oral presentation- Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats.

3. College of Health Sciences, University of Kwa-Zulu Natal, Research Symposium (year 2015)

   Oral presentation- Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats.

4. Biological Psychiatry and Neuroscience Conference (year 2015)
   (Abstract accepted for Oral presentation)

   Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats.

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Prenatal stress and early life febrile convulsions compromise hippocampal genes MeCP2/REST function in mid-adolescent life of Sprague-Dawley rats

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ABSTRACT

Early life neuronal insults exacerbate the development of febrile seizures and can result in epigenetic changes in the hippocampus. The MeCP2 and REST genes play a pivotal role in cognition as both contribute to neuronal function. In this study, cognitive function and expression of the MeCP2 and REST genes in the hippocampus were investigated in four groups of Sprague Dawley rats offspring *viz.* (1) Normally reared treated with saline (NSS). (2) Prenatally stressed treated with saline (SS). (3) Normally reared with febrile seizures (NSFS). (4) Prenatally stressed with febrile seizures (SFS). Pregnant dams were subjected to 1 h of restraint stress for 7 days starting on gestational day 14. Following birth, a once-off exposure to saline injections or febrile seizure induction was conducted on postnatal day (PND) 14. Behavioural tests were conducted using the Morris-Water maze on PND 21 and 30. Our results showed a febrile seizure effect on learning and memory in the non-stressed animals. However, febrile seizures did not exacerbate learning deficits in the prenatally stressed animals. Gene analysis found a down-regulation in MeCP2 gene expression and an up-regulation of the REST gene in prenatally stressed animals. Exposure to febrile seizure resulted in down-regulation of both MeCP2 and REST gene expression in the non-stressed animals, but febrile seizures did not exacerbate the stress effect on gene expression. This suggests that exposure to prenatal stress (SS) and febrile seizures (NSFS) may impair cognitive behavioural function. However, in the NSFS animals, there seems to be an attempt to counteract the effects of febrile seizures with time.

1. Background

Febrile convulsions are a growing concern in many facets of the health fraternity within South Africa (Ackermann & Van Toorn, 2012; Wager & Newton, 2009). A febrile seizure may be categorised by acute episodes of synchronous firing of neurons in the brain thereby altering neuronal functional balance on the central nervous system (CNS) (Qulu, Daniels, & Mabandla, 2012; Vezzani, Maroso, Balosso, Sanchez, & Tamas, 2011). Febrile convulsions are shown to be exacerbated following exposure to prenatal stress (Qulu et al., 2012) (see Figs. 1–5b).

Prenatal stress is a term used to describe the stress response experienced by pregnant women due to various external factors (environmental factors, emotional and financial implications) thereby affecting the developing foetus (Lemaire, Koehl, Le Moal, & Abrous, 2000). The mechanism of action of stress involves the release of glucocorticoids from the adrenal glands into the blood circulation of the expectant mother (Lemaire et al., 2000; Vallée et al., 1997). Glucocorticoids can easily cross the placental membrane under normal physiological conditions (Barbazanges, Piazza, Le Moal, & Maccari, 1996; Seckl, 2004). Foetal glucocorticoid concentrations are high during the last trimester, as it is required for the development of organ systems in the developing foetus (Challis et al., 2001). However, exposure to prenatal stress further exacerbates glucocorticoid release thereby impairing the negative feedback mechanism involving 11β-hydroxysteriod-dehydrogenase type 2 (11β-HSD2) (Challis et al., 2001). 11β-HSD2 is an enzyme responsible for dampening glucocorticoid effects on the developing HPA-axis in the foetus under prolonged exposure to glucocorticoids (De Kloet, Fitzsimons, Datson, Meijer, & Vreugdenhil, 2009; Harris & Seckl, 2011; Seckl, 2004). Prolonged exposure to glucocorticoids has been shown to have deleterious effects on various areas of the brain in the developing foetus (Challis et al., 2001; Champagne, de Kloet, & Joëls, 2009; McEwen, 2001). The hippocampus is known to be more susceptible

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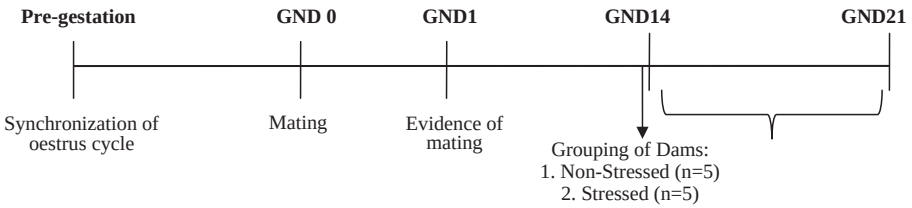

Fig. 1. Outline of prenatal handling protocol.

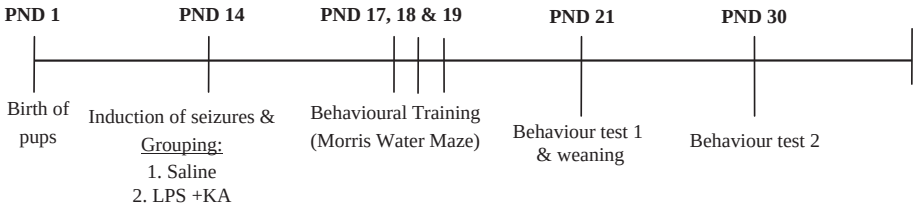

Fig. 2. Outline of post-natal handling protocol.

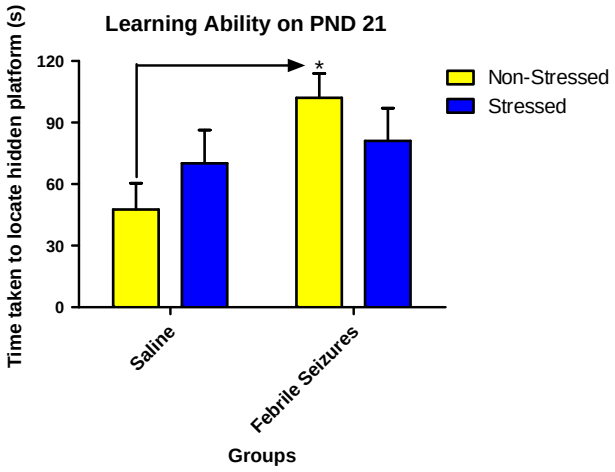

Fig. 3a. Graph displaying average time taken to locate the hidden platform in the MWM in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), $n = 6$ per group. *(NSS vs. NSFS), $p < 0.05$.

to neuronal insults in early life due to high expression of glucocorticoid receptors (Challis et al., 2001; McEwen & Sapolsky, 1995; Sapolsky, 1999).

Prenatal stress has also been linked to changes at cellular and molecular level involving DNA methylation (Fine, Zhang, & Stevens, 2014). DNA methylation has been shown to affect the hippocampus during development by multiple mechanisms *viz*., inhibition of DNA methyltransferases (DNMTs) resulting in reduced glutamate concentrations thereby affecting the transcriptional function of gene Methyl-CpG-binding protein 2 (MeCP2) (Guy, Cheval, Selfridge, & Bird, 2011; Riazi, Galic, & Pittman, 2010). MeCP2 is a common gene found in the brain that is susceptible to epigenetic related malfunctions particularly during the gestational, neonatal and pubertal stages of mammalian development (Guy et al., 2011; Qureshi & Mehler, 2010). MeCP2 functions by binding to various co-repressors (*Sin3a, NCOR, c-ski*) which activate cAMP response element-binding protein (CREB) simultaneously regulating Brain-Derived Neurotrophic Factor (BDNF) transcription (Qureshi & Mehler, 2010). MeCP2 is a multi-functional gene (transcriptional regulation, DNA repair, chromosome segregation), which makes it susceptible to mutation during neural development (Guy et al., 2011; Qureshi & Mehler, 2010). Symptoms related to MeCP2 mutations include autistic features, seizures and mental retardation (Guy et al., 2011; Qureshi & Mehler, 2010). Furthermore, Repressor Element-1 Silencing Transcription factor (REST) has been shown to be a pivotal player in epigenetics (Qureshi & Mehler, 2010). Initially it was thought that REST silences neuronal gene transcription in supporting cells, however studies have emerged showing that REST is the master regulator of neurogenesis along with gene silencing (Bruce et al., 2004). REST mediates its action of chromatin and histone modification through the recruitment of transcriptional repressors SIN3A/SIN3B and methylated CpG binding proteins; MeCP2 (Ballas & Mandel, 2005; Lunyak et al., 2002). Upon stimulation such as depolarisation, MeCP2 and other SIN3A leave the CpG site, allowing for higher level of gene expression (Ballas & Mandel, 2005).

Therefore in our study we aimed to better our understanding of the MeCP2 and REST genes in the developing brain by investigating the effects of early life exposure to neuronal insults (prenatal stress and febrile seizures) on learning and memory as well as determining the role of MeCP2/REST genes activation on hippocampal function.

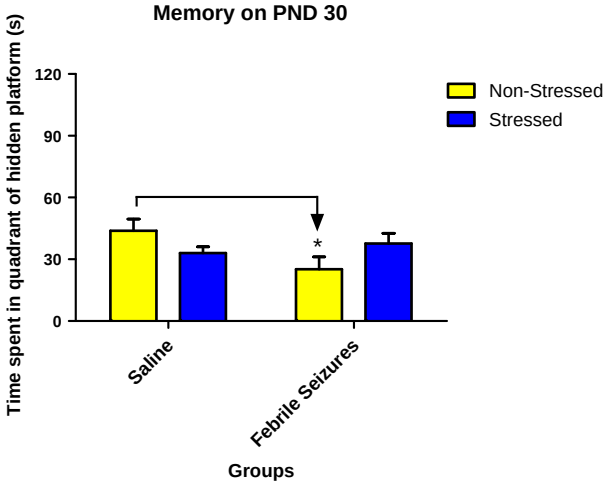

Fig. 3b. Graph displaying average time spent in the quadrant of the hidden platform in the MWM in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), $n = 6$ per group. *(NSS vs. NSFS), $p < 0.05$.

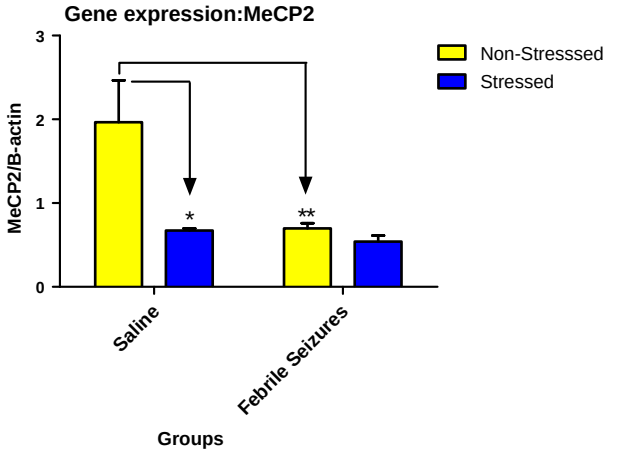

Fig. 4a. Graph displaying MeCP2 gene expression in the hippocampus of P30 rats in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), $n = 6$ per group. *(NSS vs. SS) and **(NSS vs. NSFS), $p < 0.05$.

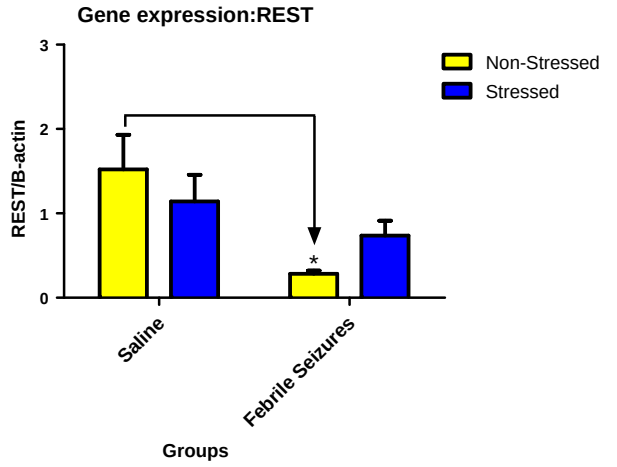

Fig. 4b. Graph displaying REST gene expression in the hippocampus of P30 rats in the following groups: non-stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), $n = 6$ per group. *(NSS vs. NSFS), $p < 0.05$.

2. Materials & methods

2.1. Animals

Sprague-Dawley rats were obtained from the Biomedical Resource Centre (BRC) of the University of KwaZulu-Natal. The animals were kept under standard BRC conditions (20% humidity at temperature of ±30 °C) and a 12 h light/dark cycle (lights on at 06h00) was maintained. Food (Rodent Ripe Pellets, Meadow, South Africa) and water were available *ad libitium*. All animals were housed in standard conventional polycarbonate 1291H techniplast cages (425 × 266 × 185 mm, floor space: 800 m^2). All experimental procedures were approved by the Animals Ethics Research Committee of the University of KwaZulu-Natal (073/14/animal) in accordance with guidelines of the National Institute of Health, USA.

2.2. Prenatal handling

2.2.1. Synchronisation

Ten female Sprague-Dawley rats were housed in pairs for a week to synchronise their oestrus cycles. The oestrus cycle of

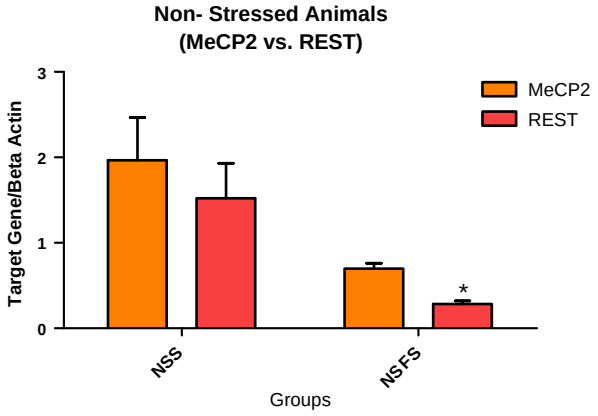

Fig. 5a. Graph displaying a comparative analysis of overall gene expression in non-stressed (NSS) and non-stressed febrile seizure (NSFS), $n = 6$ per group. *(NSFS MeCP2 vs. NSFS REST), $p < 0.05$.

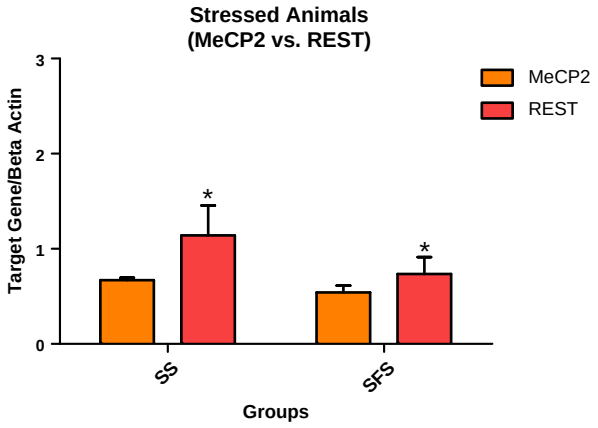

Fig. 5b. Graph displaying a comparative analysis of overall gene expression in stressed (SS) and stressed febrile seizures (SFS), $n = 6$ per group. *(SS MeCP2 vs. SS REST) and *(SFS MeCP2 vs. SFS REST), $p < 0.05$.

rodents is approximately four to five days, with each day representing 1 of 4 phases; pro-oestrus, oestrus, met-oestrus and di-oestrus. Each phase displays distinct cell maturity (Hubscher, Brooks, & Johnson, 2005; Nelson, Felicio, Randall, Sims, & Finch, 1982). Vaginal smears were taken daily to identify the pro-oestrus phase of their cycle.

2.2.2. Mating

A male Sprague-Dawley rat was introduced to a pair of female rats during their pro-oestrus phase. Vaginal smears were taken the following morning. The presence of sperm indicated successful mating and was regarded as gestational day 1 (GND 1). Following successful mating, the male rat was removed from the cage.

2.2.3. Prenatal stress protocol

On gestational day 14 (GND 14), pregnant dams were divided into 2 groups: a non-stressed and a stressed group. The non-stressed rats were left undisturbed in their home cages. The stressed rats were taken daily to a separate room and placed in

Table 1
Pups were grouped as follows.

Non-stressed animals (NS)	Stressed animals (S)
Saline (NSS)	Saline (SS)
Febrile seizure (LPS + KA) (NSFS)	Febrile seizure (LPS + KA) (SFS)

Table 2
Seizure response were scored as follows.

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

rodent restrainers for 1 h daily for 7 days starting at 09h00. The rodent restrained rats were returned to their housing room at the end of every stress period.

2.3. Post-natal handling

2.3.1. Birth of pups

Following birth, the pups remained with their dams undisturbed until PND 14 after which they were divided into the following groups ($n = 6$ per group);

The pups were removed from their dams and placed in clean cages. Each cage, housing 6 pups per experimental group was taken to the experimental room an hour prior to administration of LPS and kainic acid so as to acclimatise to the new surroundings. The dams remained in the home room, undisturbed during the duration of the experimental procedure.

2.3.2. Induction of seizures

Control animals were injected with 2 ml saline (0.9% NaCl, Adcock Ingram, South Africa) as a vehicle. To induce a febrile seizure; 0.2 ml of LPS (200 μg/kg, Sigma, USA) was injected intraperitoneally (i.p), after which pups were returned to their dams to allow for suckling and grooming. 2.5 h later, pups were once again removed from their dams and injected with 0.2 ml kainic acid (1.75 mg/kg, i.p, Sigma, USA) (Heida, Boissé, & Pittman, 2009; Qulu et al., 2012).

2.3.2.1. Assessment of febrile convulsions. The convulsive behaviour of each rat was observed for a period of 60 min and the degree of convulsive behaviour was measured (Table 1) (Heida et al., 2009; Ojewole, 2008; Vezzani et al., 2002).

Learning ability was assessed on both male and female pups on postnatal day 21 as studies have shown that there are no sex differences at this age (Peper & Dahl, 2013). However, for the memory assessment on PND 30 only male pups were used (see Table 2).

2.4. Behavioural tests

2.4.1. Morris water maze

Training in the Morris Water Maze (MWM) took place between PND 17-19. This was followed by a test phase (PND 21 and PND 30) which assessed learning and memory deficits. This test is one of many which determines the learning and recall ability of rats, and covers exploratory, navigational, spatial and contextual memory (D'Hooge & De Deyn, 2001; Garthe & Kempermann, 2013; Morris, 1984). The MWM consists of a 1 m diameter pool, comprising of 4 quadrants. Each quadrant has a cue to assist the rat in finding the hidden platform located in one of the quadrants of the pool. The method entails placing the rat in a quadrant other than where the hidden platform is located, recording the time undertaken for the rat to reach the hidden platform is considered as the animals ability to learn (D'Hooge & De Deyn, 2001; Garthe & Kempermann, 2013; Morris, 1984). The probe test is a post-test for learning which measures the ability of an animal to remember the quadrant in which the hidden platform is located. The time spent in the quadrant of the hidden platform is considered as the ability to remember (memory) (D'Hooge & De Deyn, 2001; Garthe & Kempermann, 2013; Morris, 1984).

2.4.2. Training procedure

Animals were exposed to three training sessions for a period of three days (PND 17-19). Animals were taken to the behavioural room one hour prior to training to allow for acclimatisation to the new environment. For the training procedure, each rat was placed in a quadrant, gently placed in the water, head facing the cue. The rat was given 120 s to find the hidden platform. If a rat failed to find the hidden platform in 120 s, it was physically guided by the experimenter towards the platform and was allowed 60 s to explore the platform before being returned to the home cage. Training resumed the following day for the next two days. No training took place on PND 20.

2.4.3. Testing procedure

On PND 21, animals were removed from their rooms and taken to the behavioural room one hour prior to the behavioural test to allow for animals to acclimatise. Thereafter, animals were placed in a quadrant other than the hidden platform and the time to locate the hidden platform was recorded. Animals were then returned to their cages before being returned to their room. Animals were left undisturbed from PND 22-29. On PND 30, the probe test was conducted. Prior to the test, the animals underwent the same acclimatisation procedure previously described. For the probe test, the platform was removed completely from the maze and the animals were allowed 120 s in the water. Time spent in the quadrant of the hidden platform was timed. Following the probe test, the animals were returned to their home room. Animals were sacrificed by decapitation 5 days later (PND 35), three weeks after seizure induction.

2.5. Sacrifice

On PND 35, the rats were taken to the autopsy room 1 h before decapitation. The rats were decapitated using a guillotine; trunk blood and hippocampal tissue were collected. Hippocampal tissue was weighed prior to freezing in liquid nitrogen. All tissue material was stored in a bio freezer at −80 °C.

2.6. Neurochemical analysis

2.6.1. Real-time PCR (qPCR)

Approximately 50 mg of hippocampal tissue was weighed out ($n = 6$ per group), homogenised and suspended in 400 μl of lysis buffer (Zymo Research, USA). Thereafter, samples were homogenised using a sonicator and total RNA isolation was carried out as per manufacturers guidelines (ZR RNA MiniPrep™, USA). Purification of RNA isolates were conducted using a NanoDrop. Purity of 1.5–2.01 was considered ideal for use in the construction of cDNA. cDNA synthesis was done using the iScript™ cDNA Synthesis Kit (Biorad, South Africa), processing guidelines were provided by the manufacturer. cDNA was run through the Thermocycler as per conditions stipulated in the guidelines. The Fast start SYBR green kit (Roche Diagnostics, USA) was used in accordance to the manufacturers protocol.

Primer sequences; MeCP2 gene-Forward primer: CGTCCCCTTGC CTGAAGGTTGGA, Reverse primer: CTTTCCAGCAGAGCGACCAG; REST gene-Forward primer: CAGTTGAACTGCCGTGGG, Reverse primer: CATCCGCTGTGACCGCTG; Beta actin gene-Forward primer: GCTTCTTTGCAGCTCCTTCGT, Reverse primer: CCAGCGCAGCGATATCG were reconstituted in RNA nuclease free water according to manufacturers report (Inqaba) and was added to a master mix comprising of SYBR green dye, nuclease free H_2O and $MgCl_2$.

Thereafter cDNA was added into glass capillaries and run in the Lightcycler 480 at optimised conditions.

2.7. Statistical analysis

The data was analysed using the software GraphPad Prism (version 5). Normality and Gaussian distribution were determined using the Smirnov–Kolmogorov tests. A two-way factorial analysis of variance was performed on behavioural and gene data with stress and febrile seizures as between rat factors. Significant main effects were followed by *Bonferonni* post hoc test. Differences were considered significant when p-value <0.05.

3. Results

3.1. Morris water maze: learning and memory assessment

The following groups were assessed for learning and memory deficits; non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS). In the non-stressed (NS) groups we found a febrile seizure effect, as rats that were exposed to febrile seizures took a longer time to locate the hidden platform *(NSS vs. NSFS, $F_{(1.0)} = 5.15$, $p < 0.05$).

A week later (PND 30) non-stressed with febrile seizure animals (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS) were once again assessed using the probe test. Our findings once again showed that in non-stressed (NS) animals there was a febrile seizure effect as febrile seizure exposed rats spent less time in the quadrant of the hidden platform *(NSS vs. NSFS, $F_{(1.0)} = 5.15$, $p < 0.05$).

3.2. RT-PCR: MeCP2 and REST genes

Real-time PCR was used to analyse the MeCP2 and REST genes. Groups analysed were non-stressed with saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS) animals. There was a stress effect as prenatally stressed rats showed down-regulation of the MeCP2 gene *(NSS vs. SS, $F_{(1.0)} = 4.93$, $p < 0.05$). There was a febrile seizure effect in the non-stressed rats (NSS vs. NSFS, $F_{(1.0)} = 4.93$, $p < 0.05$).

In REST gene analysis, there was a febrile seizure effect in non-stressed rats as rats exposed to febrile seizures showed down-regulation of the REST gene expression *(NSS vs. NSFS, $F_{(1.0)} = 2.32$, $p < 0.05$).

In non-stressed animals there was a significant interaction between the MeCP2 and REST genes in febrile seizure exposed animals *(NSFS MeCP2 vs. NSFS REST, $F_{(1.0)} = 14.8$, $p < 0.05$).

In stressed animals, there was a significant interaction between the MeCP2 and REST genes in saline and febrile seizure exposed animals *(SS MeCP2 vs. SS REST, $F_{(1.0)} = 3.23$, $p < 0.05$) and *(SFS MeCP2 vs. SFS REST, $F_{(1.0)} = 3.23$, $p < 0.05$).

4. Discussion

In our study we investigated the effects of stress on learning and memory and whether any effects present are influenced by the expression of the MeCP2 and REST genes. We also investigated whether exposure to febrile seizures may influence learning and memory behaviour as well as gene expression.

A febrile seizure effect was present in the non-stressed (NSFS) animals on PND 21 as these rats struggled to find the hidden platform. This effect was prolonged as rats tested 15 days post febrile seizure induction also showed a presence of memory deficits. These findings are in agreement with studies that showed that exposure to neuronal insults early in life result in a degree of cognitive impairment later in life (Chang et al., 2003). In our study we were able to observe cognitive impairment with a single episode of febrile seizure induction. These findings may be related to the neuroinflammation present in the brain due to the presence of a fever that triggers a seizure. Studies have shown that high levels of cytokine expression directly affect neuronal function *viz.* long-term potentiation, glutamate release and activation of cell-signalling pathways (Galic et al., 2008; Moisiadis & Matthews, 2014). These are related to synaptic plasticity and are therefore important in cognition. Previous studies have shown that the occurrence of a fever resulted in the release of various inflammatory markers (Galic, Riazi, & Pittman, 2012; Riazi et al., 2010). A common laboratory method to mimic a model of inflammation via fever is by injecting lipopolysaccharide (LPS), a gram negative bacterial endotoxin that triggers the activation of innate immunity via toll-like receptors (TLRs) promoting transcription of pro-inflammatory (IL-1β, IL-6, TNFα) and anti-inflammatory (IL-1 receptor antagonist, IL-10) cytokines (Galic et al., 2008; Laflamme & Rivest, 2001). It has been shown that peripheral administration of LPS results in long-term exposure to high levels of the pro-inflammatory cytokine IL-1β in circulation compromising neural function in the brain (Popoli, Yan, McEwen, & Sanacora, 2012). Acute exposure to various forms of peripheral inflammation have been shown to result in prolonged brain excitability which leads to learning and memory impairments (Riazi et al., 2010; Vezzani, 2014). This subsequently results in excess release of intracellular Ca^{2+} due to competitive binding between IL-1β and IL-1ra leading to neuronal cell death and compromised cognitive function (Peper & Dahl, 2013; Riazi et al., 2010; Vezzani, 2014).

While we did not find differences in learning deficits in the stress and febrile seizures (SFS) animals and the stressed animals without febrile seizures (SS), both groups had a tendency to struggle to find the quadrant with the hidden platform when compared to the non-stressed (NSS) animals. The absence of a difference in the time taken to find the quadrant with the hidden platform by the stressed animals induced with febrile seizures (SFS) suggests that the seizures did not exacerbate learning impairments that may have been present. Similarly on PND 30, the recall ability of the stressed groups (SS and SFS) was also impaired as these groups struggled to find the quadrant with the hidden platform when compared to the non-stressed animals without exposure to febrile seizures (NSS).

Various studies have shown that early life neuronal insults can result in chronic physiologic and neuronal alterations in later life (Galic et al., 2008; Harre, Galic, Mouihate, Noorbakhsh, & Pittman, 2008; Spencer, Heida, & Pittman, 2005). Findings from our behavioural results were confirmed by gene analysis performed on hippocampal tissue on PND 35. We investigated the roles of the MeCP2 and REST genes in learning and memory in a prenatally stressed febrile seizure rat model.

We showed that the MeCP2 gene was significantly down-regulated in prenatally stressed animals (SS) in comparison to non-stressed animals (NSS) thereby confirming the presence of a stress effect. While we could not confirm definitive impairment in the behavioural tests, possibly due to the number of animals tested, the gene expression results were more robust in showing the presence of a stress effect. This suggests that prenatal stress had a direct effect on the epigenetic modification in the developing brain. Studies have shown the deleterious effects of various stressors in early life on brain development (McEwen, 2001, 2008; McEwen & Sapolsky, 1995; Sapolsky, Krey, & McEwen, 1985). Early life stress can cause selective methylation or demethylation on specific cytosine residues in the DNA sequence which are shown to be influenced by 11β-HSD2 levels i.e. decrease in MeCP2 levels results in the decrease of the 11β-HSD2 enzyme, resulting in

compromised brain function in the foetus (Alikhani-Koopaei, Fouladkou, Frey, & Frey, 2004; Weaver et al., 2007). Therefore our findings suggest that prenatal stress exposure alone has deleterious effects on the developing brain which can result in long-term learning and memory deficits.

Non-stressed animals exposed to febrile seizures (NSFS) showed a significant down-regulation of the MeCP2 gene when compared to control animals (NSS) thereby confirming a febrile seizure effect. These results may be related to spontaneous excitatory activity that occurred in early life exposure to an acute seizure. It has been shown that a decrease in MeCP2 gene expression is due to a decrease in excitatory postsynaptic currents (EPSCs) (Dani et al., 2005). It has also been shown that the MeCP2 gene regulates the amount of glutamate receptors in hippocampal neurons (Chao, Zoghbi, & Rosenmund, 2007). In our study the acute bout of excitability due to the injection of kainic acid may have resulted in over-excitation of glutamate receptors. The injection of this glutamate receptor agonist may have influenced the down-regulation of the MeCP2 gene.

Stressed animals exposed to febrile seizures (SFS) did not exhibit learning and memory deficits when compared to control stressed animals (SS) suggesting that exposure to febrile seizures did not exacerbate the MeCP2 gene down-regulation. Stress increases arginine vasopressin in the hypothalamus which results in a decrease in MeCP2 gene expression (Murgatroyd et al., 2009). We found no change in MeCP2 expression between stressed animals with and without febrile seizure induction (SS and SFS). Therefore, exposure to a single early life stress may impair cognition but further exposure to infections resulting in febrile seizures do not exacerbate these impairments.

The REST gene regulates its expression through recruitment of various co-repressors including MeCP2 (Ballas & Mandel, 2005; Lunyak et al., 2002; Qureshi & Mehler, 2010). Therefore if MeCP2 expression increases, REST will decrease. As MeCP2 gene promotes neurogenesis and the REST gene acts as a repressor factor, an increase in the MeCP2 gene expression acts as a protective feedback mechanism (Qureshi & Mehler, 2010). Our findings have shown that there was significant up-regulation in the REST gene expression in stressed animals (SS), suggesting compromised neural plasticity. This concurs with our behavioural result in which stressed animals showed tendencies of learning and memory impairments. It has also been suggested that decreased expression of REST gene promotes cellular differentiation and neurogenesis (Ernsberger, 2012). While we did not measure neurogenesis in our study, we postulate that an increase in REST gene expression affected plasticity thereby compromising hippocampal function leading to learning and memory deficits.

A febrile seizure effect was present in non-stressed animals (NSFS) as there was a significant down-regulation of the expression of the REST gene. As behavioural results showed impaired cognitive function in these animals, down-regulation of the REST gene suggests that the hippocampal damage leading to cognitive impairment in these animals may be a transient effect. This may confirm that febrile seizures are indeed a benign condition that does not lead to permanent cognitive deficits. In order to create a febrile seizure animal model, animals were exposed to lipopolysaccharide and kainic acid. Kainic acid is a glutamate agonist that promotes increased intracellular Ca^{2+} concentrations, leading to excitotoxicity and eventually neuronal loss (Lunyak et al., 2002). Studies have previously shown that glutamate plays a role in the regulation of REST gene expression and that early life neural insults affect glutamate receptors by desensitizing the receptors after prolonged exposure to glutamate thereby compromising hippocampal cell survival (Abrajano et al., 2009; Rodenas-Ruano, Chávez, Cossio, Castillo, & Zukin, 2012). These changes in the expression of the MeCP2 and REST genes also showed a significant interaction suggesting that the relationship between both genes contributes to changes in hippocampal functioning. The inter-dependency between the two genes results in the REST gene silencing MECP2 gene expression in stressful conditions (Lunyak et al., 2002).

Stressed animals that were exposed to febrile seizures (SFS) showed no significant changes in the REST gene expression. This concurs with behavioural findings and MeCP2 gene expression results. We suggest that the damage caused by exposure to prenatal stress was great enough in hippocampal neurons, such that further exposure to a toxic insult did not further exacerbate the damage.

5. Conclusion

Our findings suggest that exposure to febrile seizures early in life affects learning and memory ability. However, it seems as if these cognitive deficits are transient as gene expression results suggests that there may be an attempt to promote neural plasticity following the febrile seizure episode. We also showed that exposure to stress early in life may affect the ability to promote neural plasticity in the hippocampus. This effect is, however, not exacerbated by further exposure to infections that lead to the development of febrile seizures.

Disclosure

We hereby disclose that there are no financial and personal conflict of interests.

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RESEARCH PAPER

Febrile seizure induction impairs neuronal plasticity in prenatally stressed rat offspring.

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Abstract

Febrile seizures in young children are a growing concern in developing economies around the world. Although this condition is regarded as benign, exposure to early life stress in these children may affect neuronal functioning in due time. We investigated whether neuronal malformation and the resulting cognitive deficits associated with stress are linked to changes in BDNF and the REST gene factor concentration and expression respectively. Furthermore, we investigated whether febrile seizure induction exacerbates the changes seen in these factors. Febrile seizures were induced on postnatal day (PND) 14 in pups of dams that were stressed during pregnancy. To assess the effects of prenatal stress on behaviour, behavioural assessments were conducted on PND 21 followed by sacrifice by decapitation. Our results show that there was a delay in the onset of seizures followed by a prolonged seizure duration in animals exposed to prenatal stress. Furthermore, prenatal stress exposure resulted in anxiety-like behaviour in young rats. We also found that prenatal stress resulted in increased hippocampal BDNF concentration, whereas exposure to febrile seizures increased REST gene expression in prenatally stressed animals. These findings suggest that exposure to prenatal stress impedes neurotrophic factor release, while accentuating the REST gene expression in febrile seizure animals. These factors may contribute to the hindering of neurogenic properties in the young, thus leading to neuronal plasticity deficits and possibly cognitive malfunction in later life.

Keywords: Anxiety-like behaviour, REST gene, BDNF, prenatal stress, febrile seizures, neural plasticity

1.1 Background

Elevation in body temperature often follows the presence of an infection in the body (Heida, *et al.*, 2004; Wager, *et al.*, 2009; Ackermann & Van Toorn, 2012). In children, infections are common triggers of seizures (Heida, *et al.*, 2004; Ackermann & Van Toorn, 2012; Wager, *et al.*, 2014). Seizures are categorised as epileptic (disorder of brain function that trigger episodic and unpredictable seizure occurrence) or non-epileptic (once-off seizure that may be evoked by electrical or chemical stimulations (McNamara, 1994). Febrile seizures are regarded as non-epileptic seizures and may be classified as a simple, complex or generalized convulsion (McNamara, 1994). Clinical findings suggest that non-epileptic convulsions lead to motor disorientation and transient cognitive deficits in patients (Hoefnagels, *et al.*, 1991). Stress has been shown to aggravate seizure progression in a kainic acid induced seizure rodent model (Qulu, *et al*., 2012). More than 10% of children who had previously been exposed to various forms of early life stressors had exacerbated seizure occurrences (Wood, *et al*., 2004). Clinical findings have further suggested that early exposure to stress affects anxiety levels thereby decreasing immune function resulting in children being more susceptible to febrile convulsions (Koh, *et al.*, 1998; Ackermann & Van Toorn, 2012).

Furthermore, it has been shown that exposure to various forms of stressors prenatally, affects the hypothalamic-pituitary-adrenal (HPA) axis particularly of the developing foetus (Qulu, *et al.*, 2012; Moisiadis, *et al.*, 2014). As a consequence, the developing neuronal circuitries in the foetus are affected due to exposure to high concentrations of circulating maternal glucocorticoids (Wilson, *et al.*, 2013; Moisiadis, *et al.*, 2014). This subsequently affects the limbic area of the brain, specifically the hippocampus, as there is a high prevalence of cortisol receptors present (McEwen, 2001; Wilson, *et al.*, 2013). Therefore, disruption in hippocampal function contributes to various anxiety disorders and cognitive deficits (McEwen, 2001; Wilson, *et al.*, 2013; Moisiadis, *et al.*, 2014).

Qulu, *et al.* (2015), has shown that prenatal stress reduces hippocampal mass in young rats. This may be a result of neuronal malformation involving various factors that involve disruption in DNA methylation affecting neurotrophic factor concentrations and multiple down- and up-stream signalling pathways altering neuronal function and structure (Qureshi & Mehler, 2010; Guy, *et al.*, 2011). Brain-derived neurotrophic factor (BDNF), a crucial player involved in neuronal plasticity, may result in the manifestation of various neuronal abnormalities and disorders when compromised (Karpova, 2014). BDNF has been closely associated with

epigenetic factors involved in DNA methylation (Karpova, 2014). Although methyl-CpG-binding proteins actively manipulate the function of BDNF, RE1-silencing transcriptional (REST) factor is essentially required to regulate transcription suggesting its involvement in neurogenesis (Pruunsild, *et al.*, 2011). It has been shown that febrile seizures have a transient effect on cognitive function by altering the balance between methyl-CpG-binding proteins and the REST gene factor in the long term (Cassim, *et al*, 2015).

Therefore the aim of this study was to investigate whether neuronal malformations and the resulting cognitive deficits associated with stress are linked to changes in BDNF concentration and the REST gene factor expression. We further investigated whether exposure to febrile seizures exacerbates the changes seen in these factors.

2.1. Material and Methods

2.1.1. Animals

Male and female Sprague-Dawley rats were obtained from the Biomedical Resource Centre (BRC) of the University of KwaZulu-Natal. The animals were housed under standard BRC conditions (20% humidity at temperature of ±23˚C) and a 12 hour light/dark cycle (lights on at 06h00) was maintained. Food and water were available *ad libitium*. All experimental procedures were approved by the Animal Ethics Research Committee of the University of KwaZulu- Natal (073/14/animal) in accordance with guidelines of the National Institute of Health, USA.

2.1.2. Prenatal Handling

a. Mating

Ten female Sprague-Dawley rats were housed in pairs for a week to synchronize their oestrus cycles (Nelson *et al*., 1982; Hubscher *et al.*, 2005). Vaginal smears were taken daily to identify when the rats were in pro-oestrus (Shorr, 1941; Moosa, *et al.*, 2014). When females reached pro-oestrus, a male rat was introduced to a pair of females, followed by the taking of vaginal smears the next morning. The presence of sperm indicated a successful mating and was regarded as gestational day (GND) 1. Following successful mating, the male rat was removed from the cage.

b. Prenatal Stress Protocol

On GND 14, pregnant dams were divided into 2 groups: a non-stressed and a stressed group. The non-stressed rats were left undisturbed in their home cages. The stressed rats were taken daily to a separate room and placed in rodent retainers for 1 hour, for 7 days starting at 09h00. The rodent restrained rats were returned to their home room at the end of every stress period (Qulu, *et al.*, 2012; Cassim, *et al*., 2015).

2.1.3. Post-Natal Handling

Following birth, the pups remained with their dams undisturbed until post-natal day (PND) 14, after which they were divided into the following groups (n=6 per group per cage);

a. Non-stressed saline (NSS)
b. Prenatally stressed saline (SS)
c. Non-stressed febrile seizure (NSFS)
d. Prenatally stressed febrile seizure (SFS)

The experimental protocol was performed in the same way as mentioned by Cassim, *et al.* (2015). Briefly, control animals were injected with 0.2 ml/g saline (0.9% NaCl, Adcock Ingram, South Africa) as a vehicle. To induce a febrile seizure (groups c and d); 0.2 ml/kg of LPS (200 µg/kg, Sigma, USA) was injected intra-peritoneally (i.p) and pups were returned to their dams to allow for suckling and grooming to avoid dehydration and hypothermia. Two and a half hours later, pups were once again removed from their dams and injected with 0.2 ml/g kainic acid (1.75 mg/kg, i.p, Sigma, USA) to induce a febrile convulsion (Heida, *et al*., 2004; Qulu, *et al.*, 2012; Cassim, *et al.*, 2015). The convulsive behaviour of each rat was observed and recorded for a period of 60 minutes. The various stages of convulsive behaviour were assessed following the guidelines provided in Heida, *et al.* (2004) and Ojewole (2008). Thereafter, pups were returned to their dams.

2.2.1. Behavioural Test

Elevated Plus Maze (EPM)

The elevated plus-maze (EPM) is a test used to assess the anxiety-like behaviour of an animal exposed to various stressors (Cannazziro, *et al.*, 2006). On PND 21, the rats were assessed for

anxiety-like behaviour using the elevated plus maze (Cannazziro, *et al.*, 2006). The test entails placing the rat in the central area of the maze facing the open arm and the degree of anxiety-like behaviour is measured by the number of entries into the preferred arm (Cannazziro, *et al.*, 2006). Animals were allowed 5 minutes in the apparatus and preference for the closed arm was regarded as a measure of anxiety-like behaviour. An entry was scored when all four paws entered into each arm.

2.2.1. Sacrifice

Rats were sacrificed by decapitation. Hippocampal tissue was collected and weighed prior to freezing in liquid nitrogen. All tissue material was stored in a bio-freezer at -80 ˚C.

2.2.3. Neurochemical Analysis

Real-Time PCR (qPCR)

The qPCR analysis was done as previously described (Cassim, *et al.*, 2015). Briefly, hippocampal tissue (50mg) was homogenized and suspended in 400µl of lysis buffer (Zymo Research, USA). This was followed by RNA extraction using the guidelines stipulated by the manufacturer (ZR RNA MiniPrep^{TM}, USA). Purification of RNA isolates was conducted using a NanoDrop. Purified RNA isolates were used in conjunction with the iScript™ cDNA Synthesis Kit (Biorad, South Africa), to construct cDNA using a Thermocycler as per conditions stipulated by the manufacturer. The Fast start SYBR green kit (Roche Diagnostics, USA), cDNA and primers were used to amplify our gene of interest at optimised conditions in the Lightcycler 480 (Roche Diagnostics, USA).

The primer sequences used:

Primer sequences;

a. REST gene-
   Forward primer: CAGTTGAACTGCCGTGGG,
   Reverse primer: CATCCGCTGTGACCGCTG;
b. Beta actin gene-
   Forward primer: GCTTCTTTGCAGCTCCTTCGT,
   Reverse primer: CCAGCGCAGCGATATCG
   (Inqaba, South Africa).

2.2.4. Immunoassay (ELISA)

BDNF protein was measured with a conventional enzyme-linked immunosorbent assay (ELISA) system. The BDNF immunoassay (Cusabio Biotech Co., Ltd., China) was performed according to the manufacturer's protocol. The harvested hippocampal tissue was rinsed and stored in 1 ml of PBS overnight at -20°C. After two freeze thaw cycles, homogenates were centrifuged at 5000xg at 7°C. The supernatant was used to determine the BDNF concentration. The sensitivity of the assay was 0.312 ng/ml- 20 ng/ml, and the cross-reactivity with other related neurotrophic factors was minimum. The intra- and inter-assay precisions were <10%, respectively.

2.2.5. Statistical Analysis

The data was analyzed using the software GraphPad Prism (version 5). Normality and Gaussian distribution were determined using the Kolmogorov-Smirnov test. A two-way factorial analysis of variance was performed with stress and febrile seizures as between rat factors. Significant main effects were followed by the *Bonferonni* post hoc test. Differences were considered significant when p- value < 0.05.

3.1. Results

3.1.1. Onset of seizure and seizure duration

Seizures were induced on PND 14 in non-stressed (NSFS) and stressed (SFS) animals. There was a stress effect as animals exposed to prenatal stress exhibited with a delay in seizure onset but had seizures of prolonged duration *(NSFS onset of seizure vs. SFS onset of seizure, $F_{(1.0)} = 132.2$, $p < 0.05$) and **(NSFS seizure duration vs. SFS seizure duration, $F_{(1.0)} = 32.6$, $p < 0.05$).

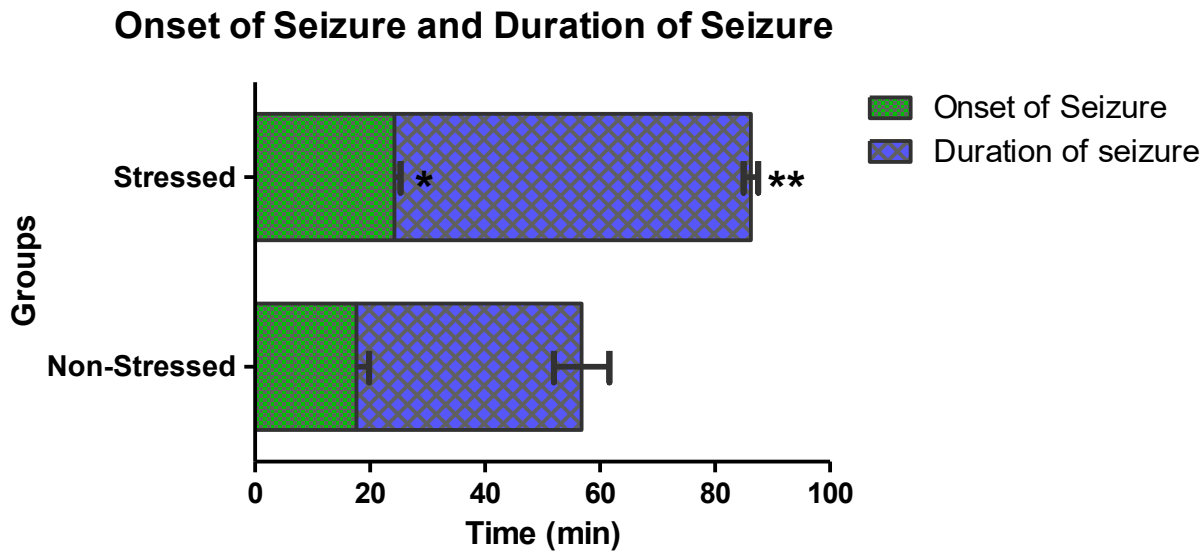

Figure 1. Graph displaying seizure onset and seizure duration in the following groups: non-stressed (NSFS) and stressed (SFS), n=6 per group.*(NSFS onset of seizure vs. SFS onset of seizure) and ** (NSFS duration of seizure vs. SFS duration of seizure), $p < 0.05$.

3.1.2. Elevated Plus Maze: Anxiety-like behaviour

Anxiety-like behaviour was assessed on the following groups of animals on PND 21: non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS). There was a stress effect in both the saline and the febrile seizure groups as these animals spent more time in the closed arms *(NSS vs. SS, $F_{(3.0)} = 97.13$, $p < 0.05$) and *(NSFS vs. SFS, $F_{(3.0)} = 97.13$, $p < 0.05$).

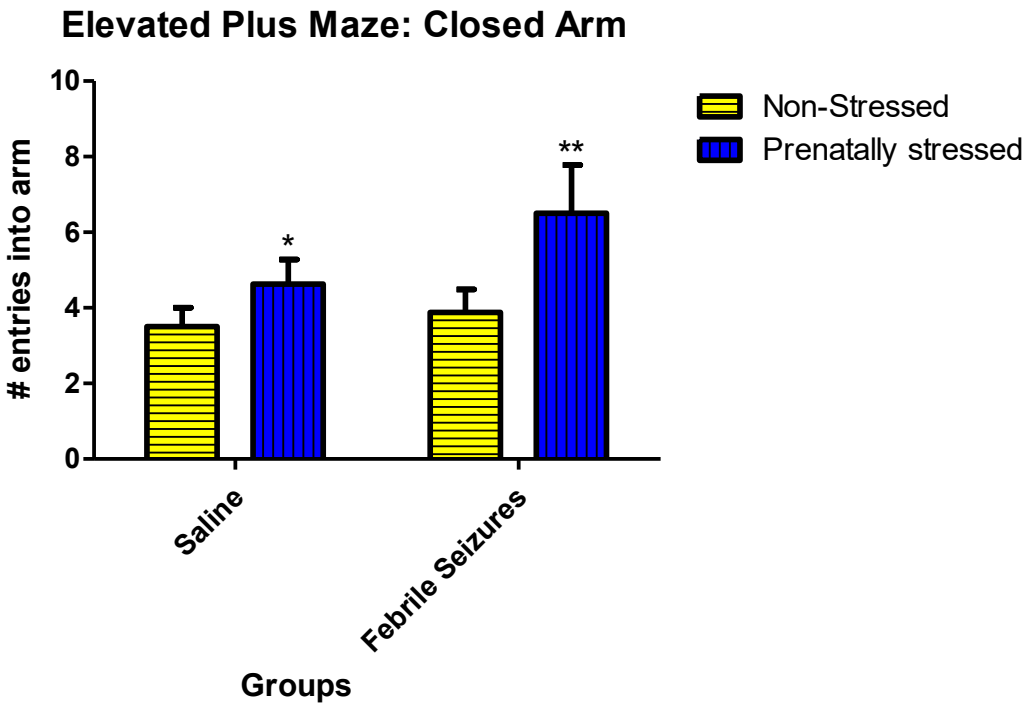

Figure 2. Graph displaying number of entries into the closed arm of the EPM in the following groups: non- stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group.*(NSS vs SS) and **(NSFS vs. SFS), $p < 0.05$.

3.1.3. Brain-Derived-Neurotrophic Factor (BDNF)

To assess a marker of neural plasticity within the hippocampus, BDNF protein concentration was measured in the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS). Animals exposed to prenatal stress showed a stress effect with high concentration of BDNF levels when compared to non-stressed animals *(NSS vs. SS, $F_{(1.0)} = 4.903$, $p < 0.05$)

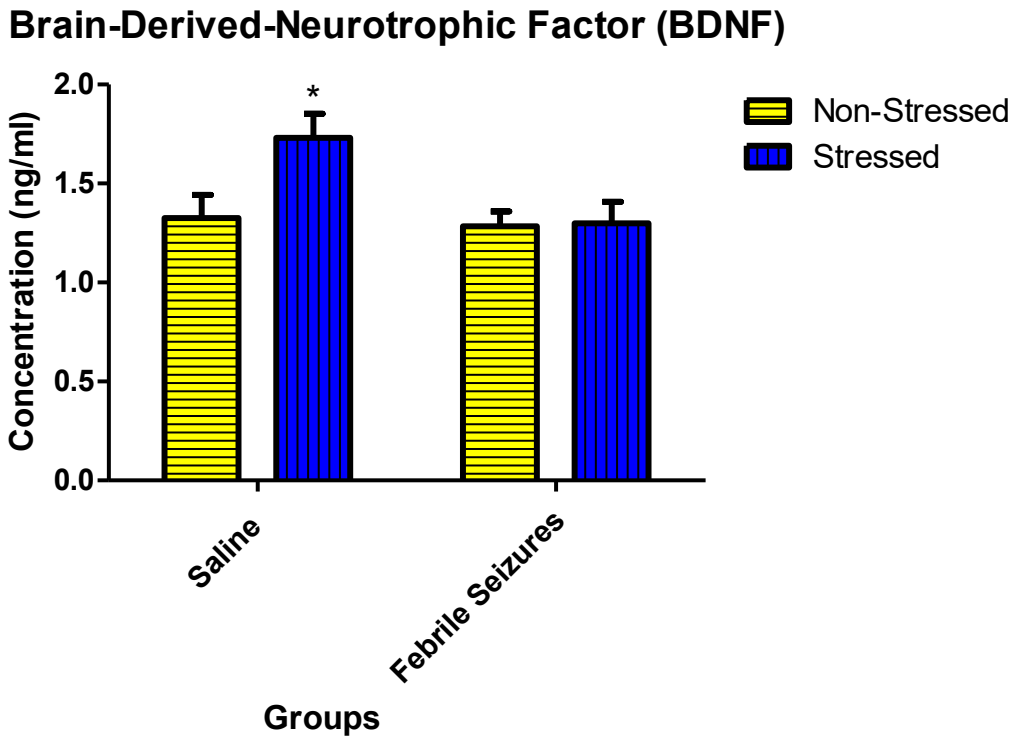

Figure 3. Graph displaying BDNF concentration in the following groups: non- stressed (NSS), non-stressed febrile seizure (NSFS), n=5 per group in duplicate. *(NSS vs SS), $p < 0.05$.

3.1.4. qPCR: REI-1-Silencing Transcription (REST)

The REST factor expression was analysed in the following groups: non-stressed saline (NSS), stressed saline (SS), non-stressed with febrile seizures (NSFS) and stressed with febrile seizures (SFS) animals.

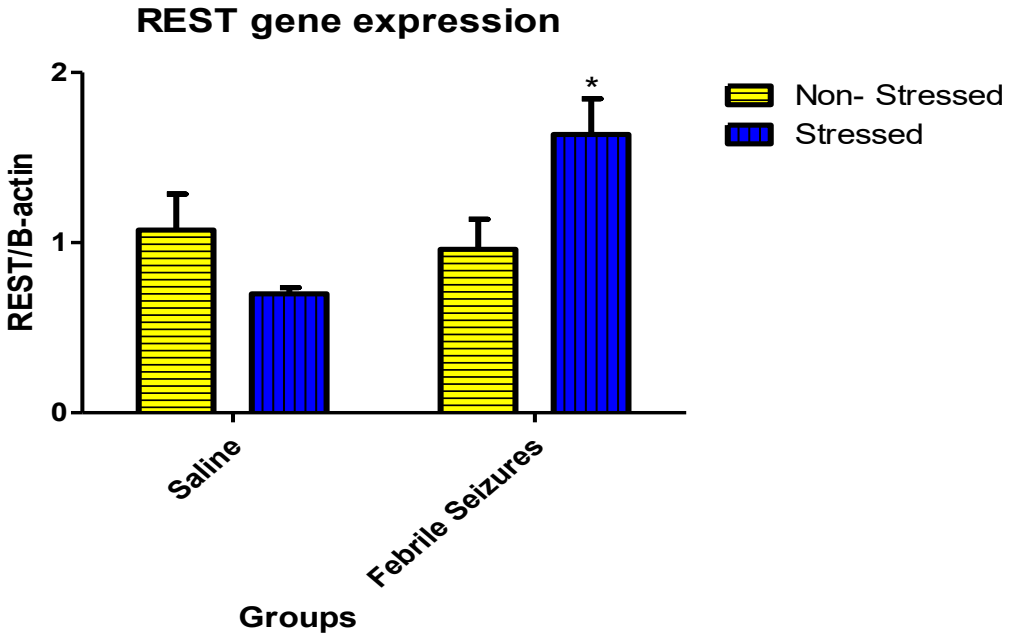

Figure 4. Graph displaying REST gene expression in the following groups: non- stressed (NSS), stressed (SS), non-stressed febrile seizure (NSFS), stressed febrile seizures (SFS), n=6 per group.*(NSFS vs. SFS), $p < 0.05$.

There was a stress effect on the REST gene expression in the febrile seizure groups *(NSFS vs. SFS, $F_{(1.0)} = 5.57$, $p < 0.05$).

4.1. Discussion

In our study we investigated the effects of stress and febrile seizure induction on young rats and whether these effects influence the concentration of BDNF and REST gene factor expression in the hippocampus.

Our findings show that animals exposed to prenatal stress had a delay in seizure onset that was followed by a prolonged seizure duration. Stress exerts its effects via various mechanisms (Weinstock, *et al.*, 2008). Exposure to pro-inflammatory cytokine release (IL-1β, TNF-α, IL-6) is a typical response to stress exposure, which increases pro-inflammatory response in the brain resulting in neuronal malfunction and subsequently altered behaviour in rodents (Qulu, *et al.*, 2012; Salim, *et al.*, 2012; Diz-Chaves, *et al.*, 2013). Studies have shown that interleukin-1β results in competitive binding to interleukin-1 receptor type 1, thereby resulting in an

increase in concentration of the neurotransmitter glutamate and an inhibition of the GABAergic system (Viviani, *et al.*, 2003). The subsequent imbalance between the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), leads to excitotoxicity due to inhibition of Ca^{2+} release causing hyperexcitability thereby exaggerating seizure duration and intensity (Viviani, *et al.*, 2003).

Furthermore, our findings showed that exposure to prenatal stress resulted in anxiety-like behaviour in the elevated plus maze (EPM). This confirms a stress effect as stressed animals showed a preference for the closed arms of the EPM. These findings are related to previous studies showing that exposure to prenatal stress results in anxiety-like behaviour (McEwen, 2007; Benoit, *et al.*, 2015). Rodents are nocturnal animals and therefore feel most comfortable in places. It has been shown that in the absence of prenatal stress, rodents are highly explorative in novel environments (Balcombe, 2006). Findings by Weinstock (2008), showed that offspring that were exposed to stress during gestational days 17-21, spent less time in the open arms of the EPM when compared to control animals. In our study, febrile seizure induction did not cause or enhance anxiety-like behaviour in the non-stressed and stressed animals. his suggests that febrile seizures may not have a prolonged effect on HPA axis activity.

Exposure to prenatal stress resulted in an increase in the concentration of BDNF in stressed animals (SS). BDNF is a crucial neurotrophic peptide that has been shown to be involved in many regulatory pathways that contribute to neuronal functioning (Calabrese, *et al.*, 2009; Yoshii & Constantine-Paton, 2009). Exposure to high levels of glucocorticoids during gestation is known to affect the feed-forward mechanism of the HPA-axis which de-sensitizes glucocorticoid receptors in the hippocampus, particularly the dentate gyrus, in brains of rodents (Boyle, 2006; McEwen, 2007; Benoit, *et al*., 2015). This subsequently promotes remodelling of the hippocampus by increasing the levels of extracellular glutamate, resulting in excitotoxic damage and possible cell death via the apoptotic pathway (McEwen, *et al.*, 2012). It is therefore possible that an increase in BDNF concentration at PND21 is an attempt to promote cell survival and regeneration in the presence of the prenatal stress effect. It has been suggested that the presence of high levels of BDNF is an attempt to promote neurogenesis which subsequently dampens the stress effect (McEwen, *et al,* 2015). This has been related to epigenetic and post-translation modifications, as acetylation and methylation have led to changes in BDNF levels resulting in anxiety-like behaviour (McEwen, *et al.*, 2015).

In non-stressed animals, febrile seizures did not affect changes in BDNF concentrations in comparison to the control (NSS) group. These findings suggest that the febrile seizure effect did not result in damage to neurons in these rats. This may be partly due to the fact that febrile seizures are a form of an acute stressor on neuronal networks (Calabrese, *et al.*, 2009) and therefore no permanent changes should be expected as neurotrophic markers aid in the "defensive pathways" to promote neurogenesis (Calabrese, *et al.*, 2009). Thus, in the stressed febrile seizure group of animals, we did not see a rise in the levels of BDNF, as these animals were primarily prenatally stressed. This suggests that prenatal stress is a form of a chronic stress and no neurogenic properties may overcome its detrimental effects.

The REST gene factor expression showed no significant change between non-stressed animals (NSS) and stressed animals (SS). However tendencies of down-regulation in the REST gene factor were seen in the stress group. While this was not a true effect (possibly due to sample size), the REST gene factor down-regulation is usually a sign of neurogenesis (Palm, *et al.*, 1998; Pruunslid, *et al.*, 2011; Cassim, *et al.*, 2015) which supports our BDNF findings.

In the febrile seizure groups a stress effect was present as there was a significant up-regulation in the REST gene factor expression in the stressed animals when compared to non-stressed animals. This suggests that stress in these animals impedes neurogenesis and neuronal plasticity, which is supported by our findings on BDNF concentration results. Palm, *et al.* (1998), showed that the REST gene factor is typically regulated during a seizure as a neuroprotective mechanism, however overexpression of this gene suggests that these animals are susceptible to cognitive impairment in later life. Furthermore, there was no change in the non-stressed febrile seizure exposed animals when compared to the control group, suggesting that our once-off febrile seizure exposure is an acute stressor with only transient effects on neuronal function. These findings reiterate that the stress effect may enhance the febrile seizure effect resulting in a delay in neurogenesis and thus may compromise cognitive function at a later stage.

5.1. Conclusion

Our findings suggest that the prenatal stress effects delay in the onset of seizures and prolongs their duration. Exposure to stress also led to an increase in BDNF concentration (non-seizure group) and an increase in the REST gene expression in rats exposed to febrile seizures. In the face of the non-elevated BDNF concentration, a known neuroprotective and neurogenic

peptide, the increase in the expression of the REST gene may suggest a repression on neural plasticity which may delay cognitive function in the affected animals.

Acknowledgements

We would like to thank the Biomedical Resource Centre for their assistance in this study. The College of Health Sciences, University of KwaZulu-Natal for their financial support. This paper forms part of a Masters degree for a co-author; Ms Sadiyah Cassim.

Disclosure

We hereby disclose that there are no known financial and personal conflict of interests in the work of this project.

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Rat brain derived neurotrophic facor (BDNF) ELISA Kit

Catalog Number. CSB-E04504r

For the quantitative determination of rat brain derived neurotrophic facor (BDNF) concentrations in serum, plasma, tissue homogenates.

This package insert must be read in its entirety before using this product.

If You Have Problems

Technical Service Contact information

Phone: 86-27-87582341
Fax: 86-27-87196150
Email: tech@cusabio.com
Web: www.cusabio.com

In order to obtain higher efficiency service, please ready to supply the lot number of the kit to us (found on the outside of the box).

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for BDNF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any BDNF present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for BDNF is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of BDNF bound in the initial step. The color development is stopped and the intensity of the color is measured.

DETECTION RANGE

0.312 ng/ml-20 ng/ml.

SENSITIVITY

The minimum detectable dose of rat BDNF is typically less than 0.078 ng/ml.
The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero standard added by their three standard deviations.

SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of rat BDNF. No significant cross-reactivity or interference between rat BDNF and analogues was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between rat BDNF and all the analogues, therefore, cross reaction may still exist.

PRECISION

Intra-assay Precision (Precision within an assay): CV%<8%
Three samples of known concentration were tested twenty times on one plate to assess.

Inter-assay Precision (Precision between assays): CV%<10%
Three samples of known concentration were tested in twenty assays to assess.

LIMITATIONS OF THE PROCEDURE

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Sample Diluent and repeat the assay.
- Any variation in Sample Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Reagents	Quantity
Assay plate (12 x 8 coated Microwells)	1(96 wells)
Standard (Freeze dried)	2
Biotin-antibody (100 x concentrate)	1 x 120 µl
HRP-avidin (100 x concentrate)	1 x 120 µl
Biotin-antibody Diluent	1 x 15 ml
HRP-avidin Diluent	1 x 15 ml
Sample Diluent	1 x 50 ml
Wash Buffer (25 x concentrate)	1 x 20 ml
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

STORAGE

<table>
<tr><td>Unopened kit</td><td colspan="2">Store at 2 - 8°C. Do not use the kit beyond the expiration date</td></tr>
<tr><td rowspan="10">Opened kit</td><td>Coated assay plate</td><td>May be stored for up to 1 month at 2 - 8°C. Try to keep it in a sealed aluminum foil bag, and avoid the damp.</td></tr>
<tr><td>Standard</td><td rowspan="3">May be stored for up to 1 month at 2 - 8° C. If don’t make recent use, better keep it store at -20℃ .</td></tr>
<tr><td>Biotin-antibody</td></tr>
<tr><td>HRP-avidin</td></tr>
<tr><td>Biotin-antibody Diluent</td><td rowspan="6">May be stored for up to 1 month at 2 - 8°C.</td></tr>
<tr><td>HRP-avidin Diluent</td></tr>
<tr><td>Sample Diluent</td></tr>
<tr><td>Wash Buffer</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

***Provided this is within the expiration date of the kit.**

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Absorbent paper for blotting the microtiter plate.
- 100 ml and 500 ml graduated cylinders.
- Deionized or distilled water.
- Pipettes and pipette tips.
- Test tubes for dilution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

- **Serum** Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
- **Plasma** Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g, 2 - 8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles. Centrifuge the sample again after thawing before the assay.
- **Tissue Homogenates** 100mg tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernate was removed and assayed immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.

Note:

1. CUSABIO is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 2-8℃, otherwise samples must be stored at -20℃ (≤1month) or -80℃ (≤2month) to avoid loss of bioactivity and contamination.
3. Grossly hemolyzed samples are not suitable for use in this assay.
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
6. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
7. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
8. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
9. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

REAGENT PREPARATION

Note:

- **Kindly use graduated containers to prepare the reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.**
- Bring all reagents to room temperature (18-25°C) before use for 30min.
- Prepare fresh standard for each assay. Use within 4 hours and discard after use.
- Making serial dilution in the wells directly is not permitted.
- Please carefully reconstitute Standards according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μl for once pipetting.
- Distilled water is recommended to be used to make the preparation for reagents or samples. Contaminated water or container for reagent preparation will influence the detection result.

1. **Biotin-antibody (1x) -** Centrifuge the vial before opening.
   Biotin-antibody requires a 100-fold dilution. A suggested 100-fold dilution is 10 μl of **Biotin-antibody** + 990 μl of **Biotin-antibody Diluent**.

2. **HRP-avidin (1x) -** Centrifuge the vial before opening.
   HRP-avidin requires a 100-fold dilution. A suggested 100-fold dilution is 10 μl of **HRP-avidin** + 990 μl of **HRP-avidin Diluent**.

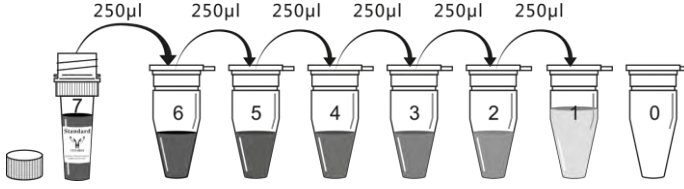
3. **Wash Buffer(1x)-** If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate (25 x) into deionized or distilled water to prepare 500 ml of Wash Buffer (1 x).

4. **Standard**

Centrifuge the standard vial at 6000-10000rpm for 30s.

Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. Do not substitute other diluents. This reconstitution produces a stock solution of 20 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 250 µl of **Sample Diluent** into each tube (S0-S6). Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (20 ng/ml). **Sample Diluent** serves as the zero standard (0 ng/ml).



Tube	S7	S6	S5	S4	S3	S2	S1	S0
ng/ml	20	10	5	2.5	1.25	0.625	0.312	0

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Add 100µl of standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
4. Remove the liquid of each well, **don't wash.**
5. Add 100µl of **Biotin-antibody (1x)** to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (**Biotin-antibody (1x)** may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)
6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating ordecanting. Invert the plate and blot it against clean paper towels.
7. Add 100µl of **HRP-avidin (1x)** to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
8. Repeat the aspiration/wash process for five times as in step 6.
9. Add 90µl of **TMB Substrate** to each well. Incubate for 15-30 minutes at 37°C. **Protect from light.**
10. Add 50µl of **Stop Solution** to each well, gently tap the plate to ensure thorough mixing.

11. Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

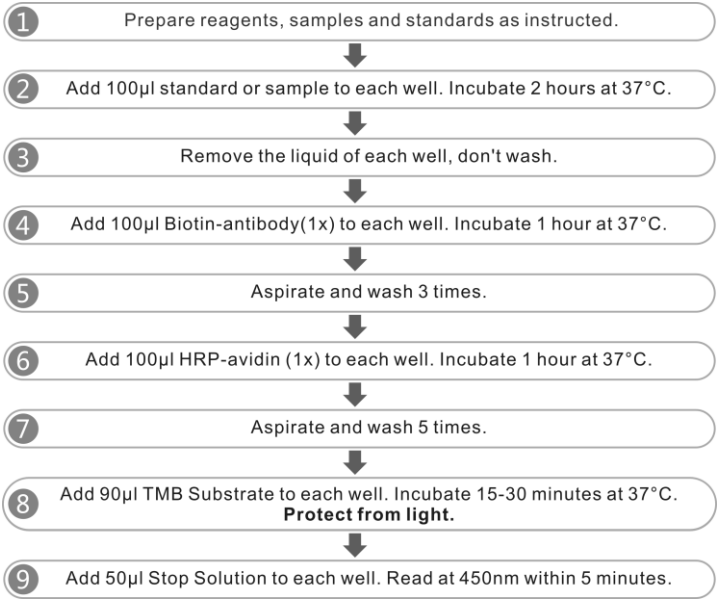
***Samples may require dilution. Please refer to Sample Preparation section.**

Note:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.
2. Samples or reagents addition: Please use the freshly prepared Standard. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.

5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), TMB Substrate should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. TMB Substrate is easily contaminated. TMB Substrate should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

ASSAY PROCEDURE SUMMARY



***Samples may require dilution. Please refer to Sample Preparation section.**

CALCULATION OF RESULTS

Using the professional soft "Curve Expert 1.3" to make a standard curve is recommended, which can be downloaded from our web.

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the BDNF concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Elabscience

www.elabscience.com

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

Rat AChE (Acetylcholinesterase) ELISA Kit

Synonyms:ACEE; ARACHE; N-ACHE; YT; acetylhydrolase

Catalog No: E-EL-R0355

96T

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

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

Phone: 86-27-87805095

Email: techsupport@elabscience.com

techelabscience@gmail.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.

Intended use

This ELISA kit applies to the in vitro quantitative determination of Rat AChE concentrations in serum, plasma and other biological fluids.

Sensitivity

The minimum detectable dose of Rat AChE is 0.469ng/mL (The sensitivity of this assay, or lowest detectable limit (LDL) was defined as the lowest protein concentration that could be differentiated from zero).

Detection Range

0.781-50ng/mL

Specificity

This kit recognizes natural and recombinant Rat AChE. No significant cross-reactivity or interference between Rat AChE and analogues was observed.

Note：

Limited by existing techniques, cross reaction may still exist, as it is impossible for us to complete the cross-reactivity detection between Rat AChE and all the analogues.

Repeatability

Coefficient of variation were<10%.

Statement: Thank you for choosing our products. This product is produced by using raw material from world-renowned manufacturer and professional manufacturing technology of ELISA kits. Please read the instructions carefully before use and check all the reagent compositions! If in doubt, please contact Elabscience Biotechnology Co., Ltd.

Storage: All the reagents in the kit should be stored according to the labels on vials. Unused wells should be returned to the foil pouch with the desiccant pack and resealed along entire edge of zip-seal. Substrate Reagent shouldn't be kept at -20℃ (Check!). Exposure of reagents to strong light should be avoided in the process of incubation and storage. All the taps of reagents should be tightened to prevent evaporation and microbial contamination. If not to store reagents according to above suggestions, erroneous results may occur.

Kit Components：

Item	Specifications	Storage
Micro ELISA Plate	8 wells ×12 strips	4℃/-20℃#
Reference Standard	2 vials	4℃/-20℃#
Reference Standard & Sample Diluent	1vial 20mL	4℃
Concentrated Biotinylated Detection Ab	1vial 120μL	4℃/-20℃#
Biotinylated Detection Ab Diluent	1vial 10mL	4℃
Concentrated HRP Conjugate	1vial 120μL	4℃(shading light)
HRP Conjugate Diluent	1vial 10mL	4℃
Concentrated Wash Buffer (25×)	1vial 30mL	4℃
Substrate Reagent	1vial 10mL	4℃(shading light)
Stop Solution	1vial 10mL	4℃
Plate Sealer	5pieces	
Manual	1 copy	
Certificate of Analysis	1 copy	

#: keep the kit at 4℃ if it's used within 30 days, keep at -20℃ for longer storage.

Test principle

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to AChE. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for AChE and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain AChE, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of AChE. You can calculate the concentration of AChE in the samples by comparing the OD of the samples to the standard curve.

Sample collection and storage

Samples should be clear and transparent and be centrifuged to remove suspended solids.

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4℃ before centrifugation for 15 minutes at 1000×g. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2 - 8℃ within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Hemolysis samples are not suitable for ELISA assay!

Cell culture supernate: Centrifuge supernate for 20 minutes to remove insoluble impurity and cell debris at 1000×g at 2 - 8°C. Collect the clear supernate and carry out the assay immediately.

Tissue homogenates: You'd better get detailed references from other literatures before assay aiming at different tissue types. For general information, hemolysis blood may affect the result, so you should mince the tissues to small pieces and rinse them in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (the volume depends on the weight of the tissue) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifugated for 5minutes at 5000×g to get the supernate.

Other biological fluids: Centrifuge samples for 20 minutes at 1000×g at 2 - 8℃. Collect the supernatant and carry out the assay immediately.

Note:

1. Samples should be used within 7 days when stored at 2-8℃, otherwise samples must be divided and stored at -20℃ (≤1month) or -80℃ (≤6months) to avoid the loss of bioactivity and contamination. Avoid repeated freeze-thaw cycles.
2. Please take the samples to room temperature (18-25℃) without extra heating before performing the assay.
3. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

Sample preparation

1. Elabscience is only responsible for the kit itself, but not for the samples consumed during the experiment. The user should calculate the possible amount of the samples needed in the whole test. Reserving sufficient samples in advance is recommended.
2. If the samples are not mentioned in this manual, a pre-experiment to determine the validity of the kit is necessary.
3. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected Elisa results due to the impacts of certain chemicals.
4. Due to the possibility of mismatching between antigen from other origins and antibodies used in

our kits, some native or recombinant proteins from other manufacturers may not be detected by our kits.

5. Influenced by factors including cell viability, cell number or sampling time, molecular from cells culture supernatant may not be detected by the kit.
6. Grossly hemolyzed samples are not suitable for use in the assay.
7. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

Other supplies required

Microplate reader with 450nm wavelength filter
High-precision transferpettor, EP tubes and disposable pipette tips
37℃ Incubator
Deionized or distilled water
Absorbent paper
Loading slot for Wash Buffer

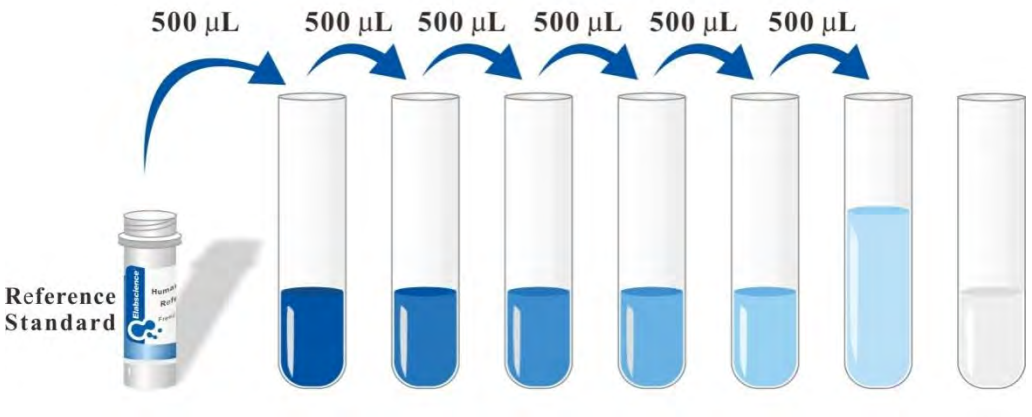
Reagent preparation

Bring all reagents to room temperature (18-25℃) before use.

Wash Buffer - Dilute 30 mL of Concentrated Wash Buffer into 750 mL of Wash Buffer with deionized or distilled water. Put unused solution back at 4℃. If crystals have formed in the concentrate, you can warm it with 40℃ water bath (Heating temperature should not exceed 50℃) and mix it gently until the crystals have completely dissolved. The solution should be cooled to room temperature before use.

Standard – Prepare standard within 15 minutes before use. Centrifuge at 10,000×g for 1 minute, and reconstitute the Standard with **1.0mL** of Reference Standard &Sample Diluent. Tighten the lid, let it stand for 10 minutes and turn it upside down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a stock solution of 50ng/mL. Then make serial dilutions as needed (making serial dilution in the wells directly is not permitted). The recommended concentrations are as follows:**50、25、12.5、6.25、3.125、1.563、0.781、0 ng/mL**. If you want to make standard solution at the concentration of 25ng/mL, you should take0.5mL standard at 50ng/mL, add it to an EP tube with 0.5mL Reference Standard &Sample Diluent, and mix it. Procedures to prepare the remained concentrations are all the same. The undiluted standard serves as the highest standard (50ng/mL). The Reference Standard &Sample Diluent serves as the zero (0 ng/mL).

(Standards can also be diluted according to the actual amount，such as 200μL/tube)



1	2	3	4	5	6	7	8	tube
50	25	12.5	6.25	3.125	1.563	0.781	0	ng/mL

Biotinylated Detection Ab – Calculate the required amount before experiment (100µL/well). In actual preparation, you should prepare 100~200µL more. Centrifuge the stock tube before use, dilute the concentrated Biotinylated Detection Ab to the working concentration using Biotinylated Detection Ab Diluent (1:100).

Concentrated HRP Conjugate – Calculate the required amount before experiment (100µL/well). In actual preparation, you should prepare 100~200µL more. Dilute the Concentrated HRP Conjugate to the working concentration using Concentrated HRP Conjugate Diluent (1:100).

Substrate Reagent: As it is sensitive to light and contaminants, so you shouldn't open the vial until you need it! The needed dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent shouldn't be dumped back into the vial again.

Note: Please don't prepare the reagent directly in the Diluent vials provided in the kit. Contaminated water or container for reagent preparation will influence the result.

Washing Procedure:

1. **Automated Washer:** Add 350µL wash buffer into each well, the interval between injection and suction should be set about 60s.
2. **Manual wash:** Add 350µLWash Buffer into each well, soak it for 1~2minutes. After the last wash, decant any remaining Wash Buffer by inverting the plate and blotting it dry by rapping it firmly against clean and toweling absorbent paper on a hard surface.

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.** It's recommended that all samples and standards be assayed in duplicate.

1. **Add Sample:** Add 100μL of Standard, Blank, or Sample per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃.
2. **Biotinylated Detection Ab:** Remove the liquid of each well, don't wash. Immediately add 100μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.
3. **Wash:** Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350μL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.
4. **HRP Conjugate:** Add 100μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37°C.
5. **Wash:** Repeat the wash process for five times as conducted in step 3.
6. **Substrate:** Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.
7. **Stop**: Add 50μLof Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.
8. **OD Measurement**: Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.
9. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

Important Note:

1. **ELISA Plate**: The just opened ELISA Plate may appear water-like substance, which is normal and will not have any impact on the experimental results.
2. **Add Sample**: The interval of sample adding between the first well and the last well should not be too long, otherwise will cause different pre-incubation time, which will significantly affect the experiment's accuracy and repeatability. For each step in the procedure, total dispensing time for

addition of reagents or samples to the assay plate should not exceed 10 minutes. Parallel measurement is recommended.

3. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Do not let the strips dry at any time during the assay. Strict compliance with the given incubation time and temperature.
4. **Washing**: The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper in the washing process. But don't put absorbent paper into reaction wells directly. Note that clear the residual liquid and fingerprint in the bottom before measurement, so as not to affect the micro-titer plate reader.
5. **Reagent Preparation**: As the volume of Concentrated Biotinylated Detection Ab and Concentrated HRP Conjugate is very small, liquid may adhere to the tube wall or tube cap when being transported. You better hand-throw it or centrifugal it for 1 minute at 1000rpm. Please pipette the solution for 4-5 times before pippeting. Please carefully reconstitute Standards, working solutions of Detection Ab and HRP Conjugate according to the instructions. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated. It is recommended to suck more than 10μL for once pipetting. Do not reuse standard solution, working solution of Detection Ab and HRP Conjugate, which have been diluted. If you need to use standard repeatedly, you can divide the standard into a small pack according to the amount of each assay, keep them at -20～-80℃ and avoid repeated freezing and thawing.
6. **Reaction Time Control:** Please control reaction time strictly following this product description!
7. **Substrate:** Substrate Solution is easily contaminated. Please protect it from light.
8. **Stop Solution:** As it is an acid solution, please pay attention to the protection of your eyes, hands, face and clothes when using this solution.
9. **Mixing:** You'd better use micro-oscillator at the lowest frequency, as sufficient and gentle mixing is particularly important to reaction result. If there is no micro-oscillator available, you can knock the ELISA plate frame gently with your finger before reaction.
10. **Security:** Please wear lab coats and latex gloves for protection. Especially detecting samples of blood or other body fluid, please perform following the national security columns of biological laboratories.
11. Do not use components from different batches of kit (washing buffer and stop solution can be an exception).
12. To avoid cross-contamination, change pipette tips between adding of each standard level, between sample adding, and between reagent adding. Also, use separate reservoirs for each reagent. **Otherwise, the results will be inaccurate!**

Calculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Create a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. It is recommended to use some professional software to do this calculation, such as curve expert 1.3 or 1.4. In the software interface, a best fitting equation of standard curve will be calculated using OD values and concentrations of standard sample. The software will calculate the concentration of samples after entering the OD value of samples. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it after appropriate dilution. The actual concentration is the calculated concentration multiplied dilution factor.

SUMMARY

1. Add 100μL standard or sample to each well. Incubate 90mintues at 37℃
2. Remove the liquid. Add 100μL Biotinylated Detection Ab. Incubate 1 hour at 37℃
3. Aspirate and wash 3 times
4. Add 100μL HRP Conjugate. Incubate 30 minutes at 37℃
5. Aspirate and wash 5 times
6. Add 90μL Substrate Reagent. Incubate 15 minutes at 37℃
7. Add 50μL Stop Solution. Read at 450nm immediately
8. Calculation of results

Troubleshooting

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by a gentle mix.
	Wells not completely aspirated	Completely aspirate wells between steps.
Low signal	Too brief incubation times	Ensure sufficient incubation time;
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation
	Improper dilution	
Deep color but low value	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
		Open the Plate Reader ahead to pre-heat
Large CV	Inaccurate pipetting	Check pipettes
High background	Concentration of detector too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions
	Stop solution not added	Stop solution should be added to each well before measurement

Declaration:

1. Limited by current conditions and scientific technology, we can't completely conduct the comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operation skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions in the description!
4. Incorrect results may led by wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for Micro-plate reader. Please read the instruction carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some unexpected reasons such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from above reasons, too.
7. Valid period: 6 months.