ASSESSMENT OF THE EFFECT OF POST-NATAL LEAD EXPOSURE ON THE HIPPOCAMPUS OF DEVELOPING WISTAR RATS

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ZARIA, NIGERIA

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN HUMAN ANATOMY

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ZARIA, NIGERIA

May, 2019

Declaration

I declare that the research work in this dissertation entitled "**Assessment of the effect of post-natal lead exposure on the hippocampus of developing Wistar rats"** has been performed by me in the Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree or diploma at this or any other Institution.

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Signature Date

Certification

This dissertation entitled "**ASSESSMENT OF THE EFFECT OF POST-NATAL LEAD EXPOSURE ON THE HIPPOCAMPUS OF DEVELOPING WISTAR RATS"** by**Oluwasegun Davis, OLATOMIDE** meets the regulations governing the award of the degree of Master of Science (M.Sc.) of the Ahmadu Bello University, and is approved for its' contribution to knowledge and literary presentation.

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Dedication

This dissertation is dedicatedto Almighty God for his infinite mercy and love that has brought me thus far. I also dedicate this work to my family most especially to my parent Mr. and Mrs. Olatomide and siblings Iyanu and Tolulope.

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I consider myself greatly privileged to express gratitude, appreciation and acknowledgement to all those who assisted me during this project work.I thank the Almighty God for his grace, mercy and faithfulness during the course of the dissertation, without him nothing would have been possible. I am indeed indebted to my supervisors, Prof. S. S. Adebisi and Dr. S. A. Musa who championed the successful completion of this dissertation through their sage advice, constructive criticisms, guidance and painstaking effort to read through the work and make corrections. I am also grateful to the Head of Department, Department of Human Anatomy, Dr. Z. M. Bauchi for her motherly support and resourcefulness in co-ordinating the affairs of the department.

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Abstract

Lead (Pb) is a highly toxic heavy metals found in every facet of environmental and biological systems. Evidence suggests that most of lead's effects on a child's central nervous system are irreversible. In this study we examined the effect of post-natal lead exposure on the hippocampus of developing Wistar rat. Nine Pregnant rats were randomly distributed into three experimental groups of three rats each, consisting of a control group (1) and experimental groups (2 and 3). After parturition, dams in Group 2 were administered 60mg/kg body weight (bwt) of lead acetate and Group 3 were administered 90mg/kg bwt of lead acetate. The pups of the dams in the experimental groups (2 and 3) were exposed to lead acetate via lactation from dams' that were administered lead (Pb) acetate via oral gavage from post-natal day (PND) 1 - PND 21.The control group (1) was given distilled water (2ml/kg bwt) throughout the experimental period. On PND 22, the pups were weighed and sacrificed after anaesthesia with 75mg/kg of ketamine. The brain tissue was excised and separated into halves along its longitudinal fissure. One half of the brain was homogenized for analysis of lead concentration via Atomic Absorption Spectrophotometry and oxidative stress markers such as Malondialdehyde (MDA), Superoxide Dismutase (SOD) and Reduced Glutathione (GSH). The hippocampus was isolated from the other half of the brain and were fixed in Bouin's fluid and paraformaldehyde fixatives for histological studies. The hippocampus was then processed routinely and stained using H and E for general cytoarchitecture, Cresyl violet stain for Nissl substance and Tomato lectin stain for microglia cells. The volume of the hippocampus and the number of activated microglia cells was then determined using stereology. The result from the present study showed a signicant decrease ($p<0.05$) in body weight,significant ($p<0.05$) increase in brain somatic index and an insignificant ($p>0.05$)decrease in brain weight of Wistar rat's pups exposed to lead acetate via lactation from PND 1-21 when compared with the control. It also revealedsignificant

($p<0.05$)increase in accumulation of lead deposit in the brain of the Wistar rat pups exposed to increasing doses of lead acetate. Lead exposure also induced oxidative stress in Wistar rat pups by causing an increase in free radicals production and a significant ($p<0.05$) decrease in the antioxidant enzymes. Neurotoxicity of lead was also evident as it caused weak staining for Nissl substanceand distortion in cytoarchitecture of the CA3 region of the hippocampus with presence of necrotic pyramidal cell, cell loss and pyknotic nucleus. In comparison with the controls, there was an insignificant ($p>0.05$) increase in the number of activated microglia cells in Wistar rat pups exposed to lead acetate via lactation from dams from PND 1-21. In conclusion, it can be inferred that exposure of Wistar rat pups to increasing doses of lead acetate from post-natal day 1-21 did not significantly trigger microglia activation but caused distortion in the cytoarchitecture of the CA3 region of the hippocampus of developing Wistar rats.

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List of Abbreviations

AAS - Atomic Absorption Spectrophotometer

AAS-L – Atomic absorption spectrophotometer- lamp standard

ABUCAUC - Ahmadu Bello University Ethics Committee on Animal Use and Care

ACCLPP - Advisory Committee on Childhood Lead Poisoning Prevention

AD - Alzheimer's disease

AP-1 - Activator protein 1

APCs – Antigen presenting cells

As- Arsenic

ATP – Adenosine triphosphate

ATPase - Adenosine triphosphatase

ATSDR-Agency for Toxic Substances and Disease Registry

BAX - BCL2 associated X protein

BBB – Blood brain barrier

BCL2 – B-cell lymphoma 2

BDNF – Brain derived neurotrophic factor

BLL – Blood lead levels

Ca- Calcium

CA – California

CA - Cornu Ammonis

CA1 – Cornu Ammonis 1

CA2 - Cornu Ammonis 2

CA3 -Cornu Ammonis 3

CA4 - Cornu Ammonis 4

CaBP – Calcium binding proteins

CAT- Catalase

CD200 –Cluster of differentiation 200

CD200R - Cluster of differentiation 200 receptor

CD40- Cluster of differentiation 40

CD91 – Cluster of differentiation 91

CDC– Center for Disease Control

CE – Coefficient of error

CNS – Central nervous system

Co- Cobalt

Cr - Chromium

Cu - Copper

CX3CL1 - C-X3-C Motif Chemokine Ligand 1

CX3CR1 - C-X3-C Motif Chemokine Receptor 1

DG– Dentate gyrus

DMT1 – Divalent metallic ion transporter 1

DNA - Deoxyribonucleic acid

DS – Developing solution

EC – Entorhinal cortex

EDTA- Ethylene-diamine-tetracetic acid

EPBS – Endogenous peroxidase blocking solution

Fe- Iron

FOXO3 – Forkhead box, class O transcription factor

GH – Growth hormone

GLT-1 - Glutamate transporter-1

GPa– Gigapascals

GPx – Glutathione peroxidase

GSH- Reduced glutathione

GSSG - Glutathione disulphide

GTPase – Guanosine trophophatase

H and E– Haematoxylin and eosin

H_2O_2– Hydrogen peroxide

HCL – Hydrochloric acid

Hg-Mercury

HMGB1 - High-mobility group B1

HO_2– Hydroperoxides

HRP – Horseradish peroxidase

HSCs – Haematopoietic stem cells

HSP60– Heat shock protein 60

HSP70 -Heat shock protein 70

HSP90 - Heat shock protein 90

ɥmol/mg protein – micromole per milligram protein

IAEA - International Atomic Energy Agency

IFN-c - Interferon-c

IGIF - Interferon gamma inducing factor

IL-10 – Interleukin-10

IL-12 – Interleukin-12

IL-18– Interleukin-18

IL-1b - Interleukin-1b

IL-1RA - interleukin-1 receptor antagonist

IL-1β– Interleukin-1 beta

IL-3 – Interleukin 3

IL-4 – Interleukin 4

IL-6 - Interleukin-6

IL-6 –Interleukin-6

INOS -Inducible nitric oxide synthase

IQ– Intelligence quotient

IRF8 - Interferon regulatory factor 8

K_2HPO_4– Dipotassium phosphate

LEC - Lateral entorhinal cortex

LSD – Least significant references

LPS – Lipopolysaccharide

LTP– Long term potentiation

MCP-1 - Monocyte chemoattractant protein-1

MDA– Malondialdehyde

MEC – Medial entorhinal cortex

Mg - Magnesium

mg/kg – Milligram/kilogram

MGluR2 - Metabotropic glutamate receptor II

MHC I – Major histocompatibility complex I

MHC II – Major histocompatibility complex II

MIP-1a- Macrophage-Inflammatory Protein-1a

MIP-1b – Macrophage-Inflammatory Protein-1b

MIP-2 - Macrophage-Inflammatory Protein-2

ml/kg – Millilitre/kilogram

mM – Millimolar

MMP-3 - Metalloproteinase-3

MRI – Magnetic resonance imaging

Na- Sodium

Na_2CO_3 –Sodium carbonate

$NAHCO_3$–Sodium bicarbonate

NARICT– National research institute for chemical technology

NF-OB - Nuclear factor kappa B

NGF - Nerve growth factor

NLRs - NOD-like receptors

nm - Nanometre

NMDA - N methyl–D-aspartate

NMDAR - N methyl–D-aspartate receptors

NO - Nitric oxide

NOS2 -Nitric oxide synthase 2

NOX1 - Nitrogen dioxide1

NOX2 - Nitrogen dioxide 2

NT-3 - Neurotrophin-3

O_2^- – Superoxide ion

P2X7 - P2X purinoceptor 7

P2X7-R - P2X purinoceptor 7 receptor

PAMPs - pathogen associated molecular patterns

Pb – Lead

Pb_3O_4 – Lead tetroxide

$PbCO_3$ – Lead carbonate

PBG - Porphobilinogen

PbS – Lead sulphide

PBS – Phosphate buffered saline

$PbSO_4$ – Lead (ii) sulphate

PG – Prostaglandin

pH- Power of hydrogen

PND – Post-natal day

PNS – Peripheral nervous system

PP – Perforant pathway

ppm – Parts per million

PRRs – Pathogen recognition receptors

PWB – Phosphate washing buffer

RAC1 - Ras-related C3 botulinum toxin substrate 1

ROS – Reactive oxygen species

RT – Room temperature

RUNX1 - Runt-related transcription factor 1

SD – Standard deviation

Se - Selenium

SEM –Standard error of mean

SH - Sulfhydryl group

SOD- Superoxide dismutase

TA-CA1 - Temporoammonic

TB – Tris-buffered solution

TBA – Thiobarbituric acid

TBARS - Thiobarbituric acid reactive substance

TBS – Tris-buffered saline solution

TBST – Tris-buffered saline solution with Triton X-100

TCA - Trichloroacetic acid

TGF-b – Tumour growth factor beta

TL – Tomato lectin

TLR - Toll-like receptor

TNF-α– Tumour necrosis factor alpha

TrkB - Tyrosine kinase B

U/ml –units per millilitre

USA – United States of America

VGCC - Voltage-dependent calcium channels

WHO – World health organization

Zn – Zinc

ZPP - Zinc protoporphyrin

δ ALA - Delta aminolevulinic acid

δ ALAD – Delta aminolevulinic acid dehydratase

µg/dl– Microgram/decilitre

µm– Micrometre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Human child development is regulated by the interactions of both endogenous and exogenous factors. One of the exogenous factors affecting early development of neurobehavior in children is the exposure to heavy metals (Wieslaw*et al.,* 2008). Human and animal populations are in constant interaction with their environment and as such are exposed to a range of chemicals and heavy metals such as lead, mercury, cadmium. Interestingly, these interactions with the environment occur through food, air and water (Burger *et al.,* 2011). Heavy metal poisoning has become an increasingly major health problem by nature of their environmental persistence, especially since the industrial revolution(Abbas*et al.,* 2002). Almost all organ systems are involved in heavy metal toxicity; however, the most susceptible systems include the nervous, renal, haematopoietic, and cardiovascular system (McDowell, 2003).

Lead (Pb) as one of the highly toxic heavy metals has been detected in every facet of environmental and biological systems (Payne, 2008; Bilandzˇic *et al.,* 2009; Clark *et al.,* 2009), particularly in industrialized cities. Lead is one of the oldest harmful agents known to mankind and has been reported to be toxic in both human and experimental animals (Goswami and Bhattacharya, 2000;Ahmad *et al.,* 2003; Loumbourdis *et al.,* 2003; Reza *et al.,* 2008) since historic times of the Greeks, Romans, Arabs and even the Egyptians. Lead exists in three forms: metallic lead, inorganic lead and organic lead (Ahamed and Siddiqui, 2007).

Human activities such as mining, manufacturing and burning fossil fuels can result in lead exposure. Lead is used in production of car batteries, hair dye, farm equipment, gasoline,

paints, ceramics, cosmetics, ammunition, water pipes, airplanes and shielding for x-ray machines (ATSDR, 2005). Despite the ban on using lead in paints or as a gasoline additive in the developed countries, human exposure to lead continues as lead does not degrade in the environment, remaining strongly absorbed by the soil. In humans, the routes of exposure include the ingestion of lead-contaminated food or drinking water containing lead leaching from older corroding pipes, inhalation in industrial settings, and dermal contact (ATSDR, 2005). Children can be exposed to peeling lead-based paint or weathered powdered paint when engaging in hobbies. Children suffering from pica (the compulsive, habitual consumption of non-food items) are particularly vulnerable (Bornschein *et al.*, 1986).

However, the magnitude of the toxic response depends on several factors, including the dose, the age of the person exposed, the life stage of a woman (childhood, lactating, menopausal), occupational exposure, duration of exposure, health and lifestyle, and nutritional status of the person exposed. Breast milk has been suggested as a significant potential source of lead exposure to nursing infants, maternal blood and bone lead levels are both important determinants of lead in breast milk (Silbergeld, 1991; Ettinger *et al.*. 2004).

Lead is readily absorbed through the gastrointestinal tract and distributes into soft body tissues such as kidney, bone marrow, liver and brain, but accumulates in the blood and bone. It is also believed that the greater absorptive capacity of the gastrointestinal tracts of children puts them at a higher risk than adults (Ab Latif *et al.*,2015). The major mechanism of lead induced toxicity in most biological systems has been reported to be via oxidative stress (Flora *et al.*, 2012). Excessive accumulation of Pb in human bodies leads to catastrophic effect on the haematopoietic, nervous, cardiovascular and renal tissues (Jacob *et al.*, 2000; Anetor, 2002; Ekong *et al.*, 2006; White *et al.*, 2007; Sansar *et al.*, 2012).

Furthermore, increasing prevalence of various abnormalities of the female reproductive system has been associated to lead exposure. Blood lead level above 10 μg/dl may adversely affect pregnancy outcome, resulting in increased risk of gestational hypertension, reduced length of gestation, miscarriage, spontaneous abortion, and preterm delivery (Torres-Sanchez *et al.,* 1999; Sowers *et al.,* 2002; Vigeh *et al.,* 2006). Indeed, no blood-lead levels appeared to be safe, because blood lead levels as low as 2.5 μg/dl in children with no observable peripheral symptoms of Pb exposure (asymptomatic) have been associated with, for example, reduced academic achievement, lower IQ, poorer memory, attention, motor dexterity and problem- solving, suggestive of altered brain development (Canfield *et al.,* 2003; Chiodo *et al.,* 2004; Lanphear *et al.,* 2005). Additionally, in mouse and rodent models, early chronic exposure to Pb resulted in abnormal motor, decreased memory and exploratory behavior (Leasure *et al.,* 2008; Azzaoui *et al.,* 2009; Kasten-Jolly *et al.,* 2012).

In vivo and *in vitro* studies have suggested that neurotoxicity may be promoted by disruption of neuroimmune system function via reduced numbers of microglia, difference in microglial cell body volume by lead exposure (Kraft and Harry, 2011; Sobin *et al.,* 2013). The neuroimmune system is comprised of microglial cells. Microglia cells are highly motile resident macrophages of the central nervous system with important roles in development, homeostasis, disease, and injury (Aguzzi *et al.,* 2013). They account for 5–20% of the total glial cell population within the central nervous system (CNS) parenchyma (Perry, 1998) and protect it through constant surveillance and scavenging of debris and foreign substances from the local environment and interacts functionally with astrocytes (Schwartz *et al.,* 2006).

Microglia keenly surveys the surrounding parenchyma via dynamic movement of their processes while the soma remains static (Nimmerjahn *et al.,* 2005) while also facilitating neuronal activity (Aloisi, 2001). They are the only cells in the CNS that originate outside the

brain. Although the origin of microglia and its cell lineage still remains highly contentious and debatable. It has been suggested that the haematopoietic stem cells in developing and adult brain transform into microglia (Alliot *et al.,* 1991; Hickey *et al.,* 1992) and the bone marrow-derived progenitors or monocytes are recruited for supplementing the microglial population. In contrast, it has also been reported that microglia also existed before brain vascularization and production of monocytes in hematopoietic tissues indicating thereby that all microglia are not haematopoietic in origin (Shepard and Zon, 2000; Takahashi 2001). Saijo and Glass (2011) reported that they arise early during development from neuroectodermal matrix cells and yolk sac cells that kernel the brain of the embryo and appear to persist throughout life.

During development, activated microglia support and protect neurite development, guide synaptic pruning, and sculpt neural circuits (Paolicelli *et al.,* 2011; Schafer *et al.,* 2012). The neuroprotective role of microglia during early development is suggested by the acute sensitivity of these cells to CNS changes, as indicated by extremely rapid activation and proliferation response times (Dissing-Olesen *et al.,* 2007). Microglia cells are activated by various agents that trigger a sequence of unique morphologic changes, including cell body enlargement. In response to these various agents, microglia activates a number of surface proteins, (CD40, MHC II), cytokines (IL-18, IL-1β, TNF-α, IL-6) and neurotoxic mediators, such as nitric oxide (NO), prostaglandin (PG) and superoxide anions (Kierdorf and Prinz, 2013). These factors initiate both repair and cytotoxic processes via interactions with other brain cells, e.g., astrocytes and neurons.

In many models of neurodegeneration and neurotoxicity, early events of synaptic degeneration and neuronal loss are accompanied by an inflammatory response including activation of microglia, perivascular monocytes, and recruitment of leukocytes and systemic macrophages (Harry and Kraft, 2012). In culture, microglia have been shown to be capable of

releasing several potentially cytotoxic substances, such as reactive oxygen intermediates, nitric oxide, proteases, arachidonic acid derivatives, excitatory amino acids, and cytokines; however, they also produce various neurotrophic factors. Microglia cells induce long-term changes within the brain because they have the capacity to become and remain sensitized (Town *et al.,* 2005; Branchi *et al.,* 2014). The sensitized state of ‘‘primed’’ microglia may be prolonged following the initial activation by stimuli, such as injury, heavy metals or stress and therefore affecting neuronal function.

Findings from *in vivo* and *in vitro* studies show that Pb exposure alters cellular functions in ways that might be expected to promote chronic microglial activation. Extensive studies have revealed that Pb exposure at low doses is extremely dangerous and can cause learning and memory impairments which is sub served by the hippocampus (Sobin *et al.,* 2013). Long term potentiation (LTP) of the hippocampal excitatory synaptic transmission is thought to be a pattern of manifestation for synaptic plasticity (Bliss and Collingridge, 1993) which is believed to be the underlying mechanisms for hippocampus-dependent learning and memory (Morris and Frey, 1997). Microglia activation can impair the learning and memory via induction of high-level expression of many factors. Either alone or in combination, these inflammatory factors may cause hippocampal neuronal injury. This neurotoxic damage may affect long term potentiation (LTP), thus leading to learning and memory deficits.

1.2 Statement of Research Problem

Lead exposure accounts for about 0.2% of all deaths and 0.6% of disabilities globally. Since the 1970s, regulation reducing lead in products has greatly reduced exposure in the developed world, however lead is still allowed in products in many developing countries (Pokras and Kneeland, 2008). Children tend to have the highest blood lead levels, possibly because at this age they breathe faster, they begin to walk and explore their environment, and they use their

mouths in their exploration (Ab Latif *et al.,* 2015). Substantial progress has been made in reducing the numbers of children exposed to higher levels of environmental lead. However, early chronic low-level lead exposure remains an unresolved child public health problem. As at October 5, 2010 at least 400 children have died from the effects of lead poisoning in Zamfara State, Nigeria (Lo *et al.,* 2010). Illegal mining activities in Niger state, Nigeria also led to disabilities and death of over 28 children.No blood-lead level is safe, because several studies have reported that blood lead levels (BLLs) as low as 2 μg/dl are associated with lower measured intelligence, poorer memory, attention andreduced neurocognitive function in children (Chiodo *et al.,* 2004; Lanphear *et al.,* 2005).While peripheral effects in adults often go away when lead exposure ceases, evidence suggests that most of lead's effects on a child's central nervous system are irreversible (Bellinger, 2004).

1.3 Justification of Study

The continuous use of lead in pesticides, water pipes, cosmetics and gasoline in developing countries necessitates a detailed investigation on its resultant effects on the brain.The activity of the hippocampus is distorted particularly in children by exposure to lead. Lead damages the cells within the hippocampus and also interferes with the release of neurotransmitters causing the disruption of communication between cells. Most studies have reported that the mechanism of lead induced neurotoxicity is via ionic mechanism and that of oxidative stress. Hence there is a need to investigate other mechanisms that may be responsible for lead induced neurotoxicity in the hippocampus. Therefore, this study was conducted to investigate the effect of post-natal lead exposure on the hippocampus of developing Wistar rats. The results from this study could help elucidate the mechanism of post-natal lead neurotoxicity in the hippocampus as well as if the effect of post-natal lead exposure on microglia activation is dose dependent, thus its effect on learning and memory.

1.4Aim of the Study

The aim of the study was to assess the effect of post-natal lead exposure on the hippocampus of developing Wistar rats**.**

1.5Objectives of the Study

The research objectives are to assess the effect of post-natal lead exposure on the:

i. body weight, brain weight and brain somatic index of the Wistar rat pups.

ii. concentration of lead deposit in the brain using Atomic Absorption Spectrophotometer-lamp standard.

iii. changes in oxidative stress markers such assuperoxide dismutase activity (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) concentration using chemical method.

iv. CA3 regions of the hippocampus of the pups using H and E and Cresyl fast violet stains.

v. cytoarchitecture of microglia in the hippocampus of the pups using Tomato lectin stain.

vi. volume of the hippocampus of the pups and number of activated microglia in the hippocampus of the pups using Cavalieri estimator and physical fractionator probe respectively.

1.6Hypothesis

Post-natal lead exposure will trigger microglia activation and cause distortion in the cytoarchitecture of the hippocampus of developing Wistar rats.

CHAPTER TWO

2.0LITERATURE REVIEW

2.1 Heavy Metals

Improvements in technology has enhanced the standards of living, but have led to new challenges with respect to ecological safety. Human safety has been threatened due to industrialization and urbanization without proper emission controls and pollution abatement (Arif *et al.,* 2015). A necessity for economic growth that relies basically on agricultural and industrial development has largely circumvented environmental protection guidelines and as such continually exposes humans to heavy metals. There is no widely agreed criterion-based definition of a heavy metal and which elements should properly be classified as such, some have based the definition on atomic weight; others, on a specific gravity of greater than 5.0 and chemical behaviour, however heavy metals are generally defined as metals with relatively high densities, atomic weights, or atomic numbers (Morris, 1992; Hawkes, 1997).

Even though heavy metals are naturally occurring in the earth's crust, exposure to them result mostly from anthropogenic activities (Figure 2.1) such as mining and smelting operations, industrial production and use, domestic and agricultural use of metals (Herawati *et al.,* 2000; He *et al.,* 2005). Significant contribution to heavy metal pollution by weathering and volcanic eruptions, metal corrosion, soil erosion of metal ions, leaching of heavy metals, Industrial sources (such as metal processing in refineries, coal burning in power plants, petroleum combustion, nuclear power stations, plastics, textiles)(Figure 2.1) have also been reported (Pacyna *et al.,* 1996; He *et al.,* 2005; Arruti, *et al.,* 2010; Sträter *et al.,* 2010).

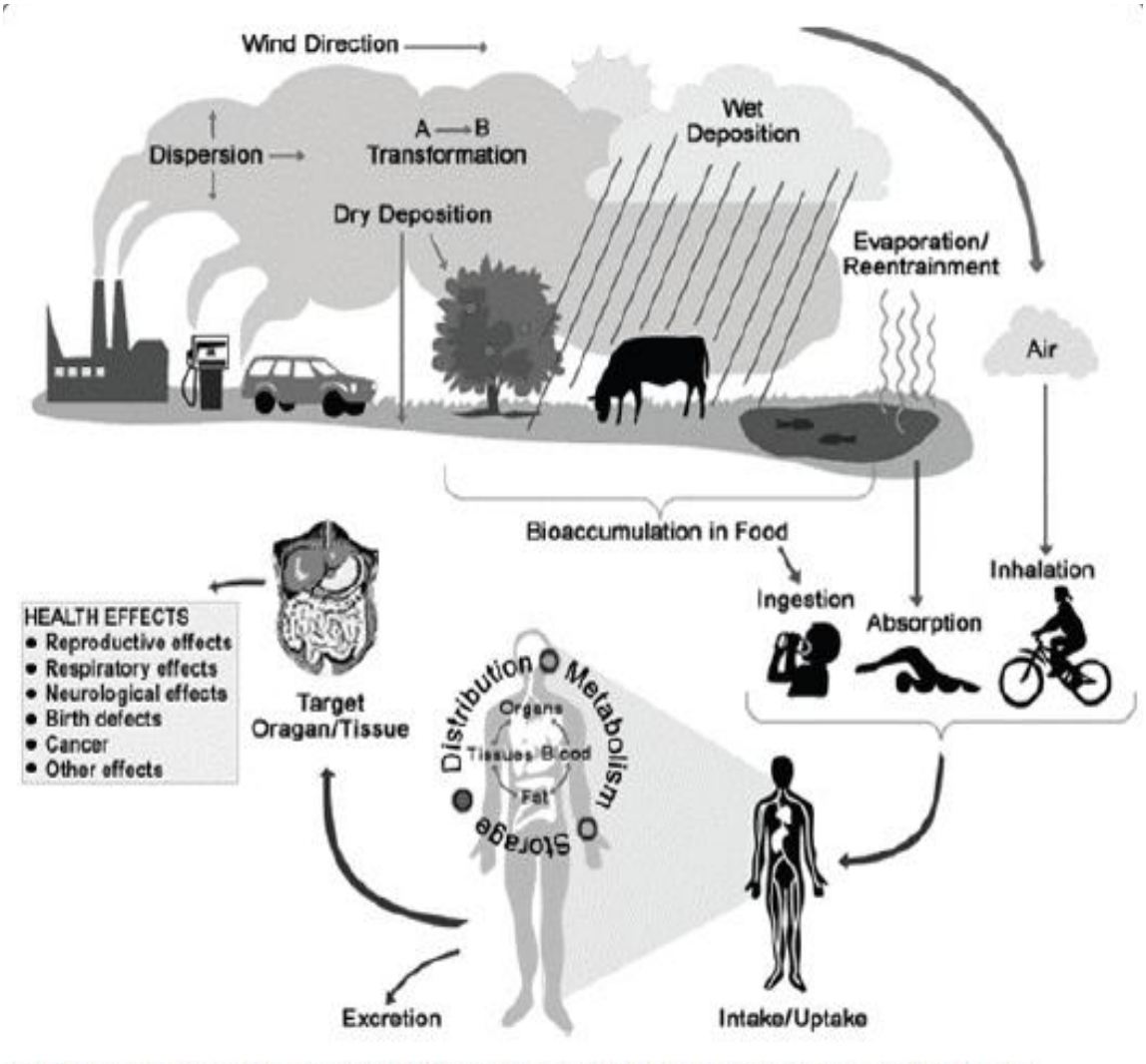


Figure 2.1: Sources of exposure to heavy metals
(Ab Latif *et al.*, 2015)

Some heavy metals are toxic when they form poisonous soluble compounds and hence have no biological role. Occasionally their chemical co-ordination and oxidation-reduction properties gives them the ability to escape control mechanisms such as homeostasis, transport, compartmentalization and binding to required cell constituents (Jaishankar *et al.*, 2014). These metals bind with protein sites by displacing original metals from their natural binding sites causing malfunctioning of cells (Flora *et al.*, 2008). Thus affecting cellular organelles and components such as mitochondria, lysosome, endoplasmic reticulum, cell membrane, nuclei, some enzymes involved in metabolism, detoxification, and damage repair, DNA (deoxynucleic acid) and nuclear proteins. This in turn causes DNA damage and conformational changes that may lead to cell cycle modulation, carcinogenesis or apoptosis (Pacyna *et al.*, 1996; Wang and Shi, 2001; Beyersmann and Hartwig, 2008).

While some heavy metals such as mercury (Hg), Lead (Pb), Arsenic(As) are identified as toxic in both trace and high quantities, some metals such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), selenium (Se) and zinc (Zn) in trace amount are essential nutrients that are required for various biochemical and physiological functions (WHO, 1996).

2.2 Lead

Lead (Pb), is a highly toxic heavy metals found in every aspect of environmental and biological systems (Payne, 2008; Bilandzˇic *et al.,* 2009; Clark *et al.,* 2009). It is primarily derived from human activities such as mining, manufacturing, and burning of fossil fuels. It is the 5th most abundant metal in the world after iron, copper, zinc and aluminium (Wong *et al.,* 1992). Lead exists in three forms: metallic lead, inorganic lead and lead compounds (or lead salts), and organic lead (containing carbon). Lead in the environment rarely occurs in its elemental state, but rather in its +2 oxidation state (Pb^{2+}) in various ores throughout the earth(Wong *et al.,* 1992). It is regarded as a potent occupational toxin and its toxicological manifestations are well known. The non-biodegradable nature of lead is the principal reason for its prolonged persistence in the environment(ATSDR, 2005).

2.2.1 Physical and chemical properties

Lead is a bluish-white heavy metalwith symbol Pb (from the Latin*plumbum*) that tarnishes to a dull gray color when exposed to air. It is soft, malleable, denser than most common materials, high resistance to corrosion and has a relatively low melting point (Lide, 2005). Lead has a Mohs hardness of 1.5 showing that it is a very soft metal and can be scratched with a fingernail (Vogel and Achilles, 2013). It has a melting point and boiling point of 327.5 °C (621.5 °F) and 1749 °C (3180 °F) respectively (Lide, 2005). It has a bulk modulus of 45.8 GPa, lesser than of aluminium (75.2 Gpa), copper (137.8 Gpa) and mild steel (160–169 Gpa)(Gale and Totemeier, 2003). It has an atomic number of 82 higher than any stable element, an atomic mass of 207, two oxidation states (+2 and +4) and four naturally occurring isotopes (^{204}Pb, ^{206}Pb, ^{207}Pb and ^{208}Pb), of which ^{208}Pb is the most abundant at 52%

of the total mass. Lead is classified as a chalcophile metallic element forming several important minerals including galena (PbS),anglesite ($PbSO_4$), cerussite($PbCO_3$) and minium (Pb_3O_4) (IAEA, 2017).

Lead belongs to group 4 of the periodic table, which also includes carbon, silicon, germanium and tin. The sum of lead's first and second ionization energies (the total energy required to remove the two 6p electrons) is close to that of tin. It has a face-centered cubic structure like the similarly sized divalent metals calcium and strontium (Christensen, 2002). It's close-packed face-centered cubic structure and high atomic weight result in a density of 11.34 g/cm^3, which is greater than that of common metals such as iron (7.87 g/cm^3), copper (8.93 g/cm^3), and zinc (7.14 g/cm^3)(Thornton *et al.,* 2001).

Lead (Pb) exists in three oxidation states: Pb, the metal; Pb (II); and Pb (IV). In the environment, lead primarily exists as Pb (II). Pb (IV) is only formed under extremely oxidizing conditions and inorganic Pb (IV) compounds are not found under ordinary environmental conditions. Lead in fine powdered form is pyrophoric (Bretherick, 2016), and burns with a bluish-white flame (Harbison *et al.,* 2015). When exposed to air and water, films of lead sulfate, lead oxides, and lead carbonates are formed; these films act as a protective barrier that slows or stops corrosion of the underlying metal. It is amphoteric, forming plumbous and plumbic salts in acid and plumbites and plumbates in alkali. It is positioned slightly above hydrogen in the electromotive series and therefore should theoretically replace hydrogen in acids (Thürmer *et al.,* 2002).

2.2.2 History of lead

Lead is one of the seven metals known to man in time immemorial. Metallic lead beads dating back to 7000–6500 BCE was found in Asia Minor and may represent the first example

of metal smelting. At that time lead had few applications due to its softness and dull appearance (Rich, 1994). The major reason for the spread of lead production was because of its relative ease of extraction as a metal. The Ancient Egyptians were the first to use lead minerals in cosmetics, before it spread to Ancient Greece and beyond. Various civilizations of the Fertile Crescentused lead as a writing material, as currency, and for construction. Lead was used in the Ancient Chinese royal court as a stimulant, currency, and as a contraceptive;Mesoamericans used it for making amulets; and the eastern and southern African peoples used lead in wire drawing. (Winder, 1993; Yu and Yu, 2004).

Rome's territorial expansion in Europe and across the Mediterranean, and its development of mining, led to it becoming the greatest producer of lead during the classical era, with an estimated annual output peaking at 80,000 tonnes. The Roman Empire used lead in the manufacturing of kitchen utensils and decorative articles, in plumbing, tableware, and other products.Lead was mostly obtained as a by-product of silver smelting just like their predecessors. Lead mining also took place in Central Europe, Britain, the Balkans, Greece, Anatolia, and Hispania, the latter accounting for 40% of world production (Hong *et al.*, 1994).

After the fall of Rome, Lead production declined drastically until the Industrial Revolution. Its high density, low melting point, ductility and relative inertness to oxidation combined with its relative abundance and low cost, resulted in its extensive use in construction, plumbing, batteries, leaded gasoline, white paints, bullets and shot, weights, solders and radiation shieldingand others (Thornton *et al.*, 2001). In the second half of the 18th century, Britain, and later continental Europe and the United States, experienced the Industrial Revolution. This was the first time during which production rates exceeded those of Rome (Hong *et al.*, 1994). Britain was the leading producer, losing this status by the mid-19th

century with the depletion of its mines and the development of lead mining in Germany, Spain, and the United States. A great share of the demand for lead came from plumbing and painting because lead paints were in regular use (Riva*et al.*, 2012).

In the late 19th century, lead's toxicity was recognized, at this time, more working class people were exposed to the metal and lead poisoning cases escalated and its use was phased out of many applications(Pokras and Kneeland, 2008). Due to its non-biodegradable nature and continuous use in paints or as a gasoline additive, and other materials, its concentration accumulates in the environment with increasing hazards. Acute exposure can cause loss of appetite, headache, hypertension, abdominal pain, renal dysfunction, fatigue, sleeplessness, while chronic exposure can result in intellectual disability, birth defects, psychosis, autism, allergies, dyslexia, weight loss, hyperactivity, paralysis, muscular weakness, brain damage, kidney damage, and even death (Papanikolaou*et al.*,2005).

2.2.3 Routes of exposure and absorption of Lead

Human exposure to lead and its compounds occurs mostly in lead related occupations with various sources like leaded gasoline, industrial processes such as smelting of lead and its combustion, lead-based paint in older homes, contaminated soil, household dust, drinking water, lead crystal, and lead-glazed pottery (Ab Latif *et al.*, 2015). Children can be exposed to peeling lead-based paint or weathered powdered paint when engaging in hobbies or activities that increase exposure (Figure 2.2). Children suffering with pica are particularly susceptible to exposure (Bornschein *et al.*, 1986).



Figure 2.2: Routes of lead exposure
(Papanikolaou *et al.*,2005)

Exposure could also occur through inhalation in industrial settings and ingestion of lead-contaminated food or drinking water containing lead leaching from older corroding pipes and fittings. Essentially, 75% of airborne Pb particles are less than 0.7 μm in diameter(Ab Latif *et al.,* 2015). Because of this small size, a large fraction of inhaled Pb may be deposited in the lungs (ATSDR, 2005). For adults with occupational exposure, the most significant route for absorption is through the respiratory tract. The percentage of inhaled lead reaching the bloodstream is estimated to be 30–40%. (Markowitz, 2000; Papanikolaou*et al.*,2005). Although toxicity of lead from industrial settings has been relatively controlled, it remains a persistent toxicant worldwide (Azizi and Azizi, 2010). Lead is readily absorbed through the gastrointestinal tract and distributes into soft body tissues such as kidney, bone marrow, liver and brain, but accumulates in the blood and bone. Absorption of lead through the intestines depends on its physical and chemical properties, age of individuals (Karrari *et al.*,2012). Compared to adults, children are more predisposed to its toxicity due the greater absorptive capacity of their gastrointestinal tracts (Ziegler *et al.*,1978; CDC, 2005).

Therefore, while adults absorb an average of 10 to 15% of the ingested quantity, this amount can increase to 50% in infants, young children and pregnant women (Markowitz, 2000; Papanikolaou *et al.*,2005). Absorption through the gut is the predominant route for children and increases with low dietary consumptions of iron, calcium, phosphorus, or zinc (Markowitz, 2000; Papanikolaou *et al.*,2005). Transcutaneous absorption of lead is little when inorganic lead compounds, such as those found in paint, are applied to the skin. In

contrast, organic (tetraethyl) lead, which is that found in gasoline, can be absorbed via the skin(Ab Latif *et al.,* 2015).

2.2.4 Mechanism of toxicity

Several studies have revealed that Lead metal causes toxicity in living cells by ionic mechanism and oxidative stress (Flora *et al.,* 2012;Wadhwa *et al.,* 2012).

2.2.4.1Oxidative stress

Under physiological conditions, there is a balance between free radicals production and antioxidant defence mechanisms which involves mostly specific enzymes such as SOD, CAT, and GPx that scavenge free radicals such as vitamins and thiol groups (SH). Oxidative stress in living cells is caused by the imbalance between the production of free radicals and the generation of antioxidants to detoxify the reactive intermediates or to repair the resulting damage (Ahamed and Siddiqui, 2007; Jaishankar*et al.,* 2014). Under the influence of lead, onset of oxidative stress occurs on account of two different pathways operating simultaneously; (i) inhibition of δ-ALAD which leads to accumulation of its substrate δ-ALA that can be rapidly oxidized to generate free radicals such as superoxide ion (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2) (Gurer-Orhan *et al.,* 2004), and lead per se has the capacity to stimulate ferrous ion that initiates membrane lipid peroxidation (Ahamed and Siddiqui, 2007)(ii)the depletion of antioxidant reserves (Figure 2.3)(Gagan *et al.,* 2012). Antioxidants such asglutathione, present in the cell protect it from free radicals such as H_2O_2.However,under the influence of lead, the level of the reactive oxygen species (ROS) increases and the level of antioxidants decreases(Figure 2.3)(Jaishankar*et al.,* 2014).

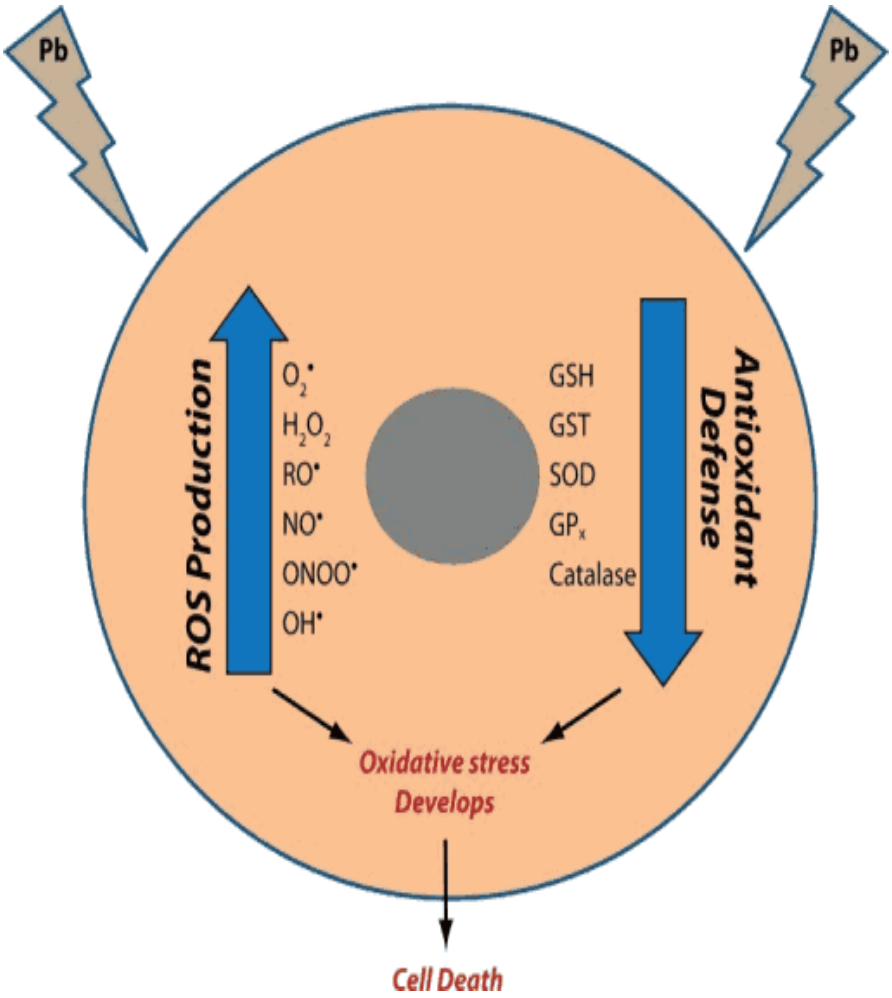


Figure 2.3: Oxidative stress mechanism of lead toxicity
(Gagan *et al.*, 2012)

At very high concentrations, ROS may cause structural damage to cells, proteins, nucleic acid, membranes and lipids, resulting in a stressed situation at cellular level (Mathew *et al.*, 2011). Neurons are particularly vulnerable to increases in ROS levels because they have a reduced capacity to detoxify ROS (Dringen *et al.*, 2005). Oxidative stress kills neurons by stimulating the Forkhead box, class O transcription factor FOXO3, a pivotal player in cell death/life pathways (Gilley *et al.,* 2003). Neurons in the hippocampal CA1 region are particularly sensitive to oxidative stress.For this reason, several antioxidant enzymes like superoxide dismutase (SOD), Malondialdehyde (MDA), Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR),reduced glutathione (GSH) and glutathione disulphide (GSSG) are the most commonly used parameters to evaluate lead-induced oxidative damage (Iliyasu *et al.*, 2015).

Superoxide dismutase (SOD) isa metalloprotein and accomplishes its antioxidant functions by enzymatically detoxifying the superoxide radical (O_2^-). Superoxide dismutase effectively catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide, which is, in turn, catabolised by catalase (located in the peroxisome) and peroxidase into oxygen and water. Lead blocks the sulfhydryl groups of SOD and is able to replace zinc ions which serve as important co-factors for the antioxidant enzymes thereby inactivating them (Flora *et al.*, 2012). Different responses of SOD have been described in animals exposed to Pb including increases, decreases, or no changes in its activity, depending on dose, length of treatment, and the target organDifferent responses of SOD have been described in animals exposed to Pb including increases, decreases, or no changes in its activity (El-Sokkary *et al.,* 2005)

depending on dose, length of treatment, and the target organ.. Adler *et al.,* (1993) reported an inhibition of SOD activity by lead in an in vitro study and indicated that this effect of lead can lead to decreased scavenging of reactive oxygen species (ROS) and result in oxidative damage. Sarath *et al.,* (2007) also reported significant decrease in SOD activity in the hippocampus of all groups treated with lead acetate with highest decrement observed in the group exposed to the highest dosage of 500ppm for 21 days.

Reduced glutathione (GSH) is a tripeptide containing cysteine that has a reactive –SH group with reductive potency. It is the most abundant intracellular thiol-based antioxidant, prevalent in millimolar concentrations in all living aerobic cells, and plays an important role in the cellular defence cascade against oxidative injury (Van Bladeren, 2000). It serves to detoxify some endogenic and exogenic compounds with conjugation reactions catalyzed by glutathione S-transferases(Van Bladeren, 2000). GSH is a cofactor for glutathione peroxidase, which catalyzes the reduction of hydrogen peroxide to water and oxygen, hence limiting the formation of hydroxyl radical, the highly toxic reactive oxygen species (Günfer *et al.,*2006). It can act as a non-enzymatic antioxidant by direct interaction of the –SH group with ROS, or it can be involved in the enzymatic detoxification reactions for ROS, as a cofactor or a coenzyme. It possesses carboxylic acid groups, an amino group, a –SH group, and two peptide linkages as sites for reactions of metals. Lead binds exclusively to the –SH group, which decreases the GSH levels and can interfere with the antioxidant activity of GSH (Ahamed and Siddiqui, 2007). Günfer *et al.,* (2006) reported a decrease in serum GSH levels of Wistar rats administered with aluminium.A decrease in GSH levels in the kidneys was also reported by Ponce-Canchihuamán *et al.,* (2010) after intraperitoneal administration of 25 mg/0.5 mL of lead acetate weekly.

Lead has been reported to have toxic effects on cell membrane structure and functions. The presence of double bonds in the fatty acid of the cell membrane weakens the C–H bonds on

the carbon atom adjacent to the double bonds and makes hydrogen removal easier. Therefore, fatty acids containing zero to two double bonds are more resistant to oxidative stress than are the polyunsaturated fatty acids withmore than two double bonds(Ahamed and Siddiqui, 2007). Because fatty acid chain length and unsaturationare associated with membrane susceptibility toperoxidation, lead-induced arachidonic acid elongation might be responsible for the enhanced lipid peroxidation in the membrane. By causing lateral phase separation and/or by increasing lipid peroxidation rates, lead could affect membrane related processes such as the activity of membrane enzymes, endo- and exocytosis, the transport of solutes across the bilayer, and signal transduction processes (Ahamed and Siddiqui, 2007). The most common group of indices used to assess oxidative stress is that of peroxidation products of lipids, usually polyunsaturated fatty acids, which are susceptible to free radical attack (Lakshmi *et al.*, 2013). Many studies have showed that MDA, the most used end-product of lipid peroxidation, will be increased with Pb treatment (Xia *et al.,* 2010; Lakshmi *et al.*, 2013). Bennet *et al.*,(2007) and Abdel Moneim, (2016) reported elevated MDA levels in brains and livers of lead acetate treated rats respectively. Alya *et al.,* (2015) also reported MDA amplification in the liver and kidney after exposure to lead (Pb).

2.2.4.2 Ionic mechanism

The ionic mechanism of lead toxicity occurs mainly due to the ability of lead metal ions to replace other monovalent and bivalent cations like Na^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+} which ultimately disturbs the biological metabolism of the cell. These ionic mechanism causes significant changes in various biological processes such as cell adhesion, maturation, apoptosis, ionic transportation, protein folding, enzyme regulation, and release of neurotransmitters. Most of the effects of lead toxicity are due to its similarity with Ca^{2+} (Jaishankar*et al.,* 2014). Lead ion (Pb^{2+}) can substitute calcium even in picomolar concentration and can interfere with many intracellular processes in which Ca^{2+} is involved, for example, as a second messenger

(Bressler *et al.,* 1999). Pb^{2+} via several mechanisms can interfere with ion channels, receptors and transporters, altering intracellular Ca^{2+} homeostasis, and these actions can explain, in part, the pathogenesis of lead poisoning and its production of symptoms in animals and humans (Audesirk, 1993; Atchison, 2003).

Neurotoxicity of lead exposure has been widely studied and many ion channels, receptors, transporters and pumps have been directly or indirectly implicated in lead toxic effects (Kursula and Majava 2007; Neal and Guilarte 2010; Baranowska-Bosiacka *et al.,* 2012).It has been reported, mostly from experiments in isolated neurons, that voltage-dependent calcium channels (VGCC) can be directly affected by lead ion exposure (Peng *et al.*, 2002; Atchison, 2003). In addition, lead ions can directly bind to calmodulin (Wilson and Brunger 2003; Kursula and Majava, 2007), which has been implicated in the ion-dependent inactivation of L-type calcium channels.

N methyl–D-aspartate receptors (NMDAR) are known to be inhibited by exposure to lead, resulting in memory problems related to interruption of long term potentiation which limits the permanent intake and storage of newly learned knowledge sub-served by the hippocampus (Flora *et al.,* 2012; Karri *et al.,* 2016). Voltage-gated sodium channels in hippocampal CA1 neurons have also been shown to be inhibited and inactivated by Pb^{2+} concentrations around 100 μm (Gu *et al.,* 2005; Yan, 2008). Lead ions have also been reported to reversibly inhibit delayed and transient outward potassium currents in hippocampal neurons (Yu *et al.,* 2003).

2.2.5 Distribution of lead after absorption

Once absorbed it is transported by the bloodstream to other tissues. Lead accumulates majorly in the blood, soft tissues, and bone. In blood, approximately 99% of the lead is found

in the erythrocytes, leaving about 1% in the plasma and serum (Rabinowitz, 1991). The concentration of lead in plasma is more significant than that in whole blood because it determines the amount of lead distributed to target organs, i.e. brain, lungs, spleen, renal cortex, aorta, teeth, and bones (Papanikolaou, *et al.,*2005). The transfer of lead from blood to soft tissues is low and takes approximately 4 to 6 weeks (Papanikolaou *et al.,*2005). In blood has an estimated half-life of 35 days, in soft tissue 40 days, and in bones 20 to 30 years (Roberts *et al.,* 2001; Papanikolaou *et al.,*2005). This increases in the case of pregnant women and of children whose bones are in a developing stage. The developing bones in children which undergo remodelling allow the lead to be continuously reintroduced into the blood stream (Barbosa *et al.,* 2005).

The initial distribution of lead throughout the body is dependent on blood flow to the tissues. More than 95% of lead is deposited in skeletal bone as insoluble phosphate (Papanikolaou *et al.,*2005). It has been determined that lead crosses the placental barrier, with fetal uptake, beginning at 12 weeks of gestation and continuing throughout development up to birth. Concentrations of lead in umbilical cord blood were found to be 80–100% of the maternal blood lead level (Papanikolaou *et al.,*2005). Bone to blood mobilization increases during pregnancy, lactation, physiological stress, chronic disease, along with advanced age (Gulson, *et al.,*2003). Its release back into the bloodstream, particularly during times of calcium stress in the pregnancy period makes the developing foetus more prone to its toxicity through mobilisation as part of the blood supply and after birth through lactation of the infant. Elevated levels in pregnant women often lead to preterm labour, miscarriages, spontaneous abortion or still births and low birth weight children (Gulson*et al.,*2003).

Some studies have highlighted the involvement of Pb in the disruption of body weights in animals exposed to this metal. The work of Ronis *et al.,* (1998) reported that the treatment of

pregnant rats at doses of Pb between 0.05% and 0.45% w/v caused disturbances in pups' body weight at birth, and growth suppression during the pre-puberty and the puberty periods by causing a decrease in the plasma concentration of IGF (Insulin-like growth factor) and the rate of pituitary growth hormone (GH) production.Aprioku and Siminialayi, (2013) also reported a significant and time-dependent reductions in body weight after perinatal exposure to 4 or 8 mg/kg lead acetate respectively. In a related study, weight reductions was also reported in neonates of female rats that received daily injections of 5 mg/kg lead acetate at different stages of pregnancy (Chandra *et al.,* 1983). A decrease in body weight may be as a result of decline in the consumption of food (anorexia) which is induced by heavy metal consumption. Another possible explanation for the loss of body weight may be the decreased muscle mass and cachexia due to the oxidative stress induced by lead (Zaheer *et al.*, 2013).

2.2.6 Excretion of lead

Generally, excretion of lead is low, with the chief route of excretion being via the urinary tract (Philip and Gerson,1994). The use of chelating agents can improve lead excretion in urine and this constitutes the basis of the therapeutic approach to lead poisoning (Papanikolaou *et al.,* 2005). Inorganic lead is not digested; however, alkyl lead compounds are oxidized by the hepatic P 450 system. Lead may also be excreted with bile through the gastrointestinal tract (Philip and Gerson,1994). Although small amounts of lead are excreted through the sweat and the nails, these routes do not have any practical significance. In general, lead is excreted extremely slowly from the body, with its biological half-life estimated at 10 years, thus facilitating accumulation in the body (Papanikolaou *et al.*,2005).

2.2.7 Effect of lead on body systems

2.2.7.1Effect of lead on the haematopoietic system

The adverse effects of lead appear even with blood concentrations as low as 10 μg/dl. It produces its effect in the blood by interfering with the biosynthesis of heme (Skoczynska**,** 2008). Because of its high affinity for thiol (–SH) groups, it affects the functioning of δ aminolevulinic acid dehydratase (δ ALAD), by binding to the SH-groups of proteins or by displacing some essential metal ions. The δ-aminolevulinic acid dehydratase is the second enzyme of the heme pathway that catalyzes the condensation of two molecules of δ-aminolevulinic acid (δ ALA) to form the monopyrrole porphobilinogen (PBG) (Keladа*et al.,*2001). Inhibition of the activity of δ ALAD causes accumulation of δ ALA in blood which enhances and prolongs microglial activation (Kaushal *et al.,* 2007) and has been associated with oxidative damage by causing formation of reactive oxygen species (Ahamed *et al.,* 2006; Patrick, 2006; Martínez *et al.,* 2013). In addition, it prevents incorporation of Fe^{2+} into the protoporphyrin molecule, accompanied by binding of zinc (Zn^{2+}) rather than Fe^{2+} into the protoporphyrin ring, thereby producing zinc protoporphyrin (ZPP). Presence of δ ALA or ZPP in blood or in urine is considered a potent marker for early biogenic effect of lead in humans.

2.2.7.2Effect of lead on the cardiovascular system

Both chronic and acute lead poisoning causes cardiac and vascular damage with potentially lethal consequences including hypertension and cardiovascular disease (Navas-Acien *et al.,* 2007). Low level lead exposure can contribute to hypertension in both animals and humans (ATSDR, 2005). Other major disorders include ischemic coronary heart disease, cerebrovascular accidents and peripheral vascular disease (ATSDR, 2005).

2.2.7.3Effect of lead on the reproductive system

Lead causes a number of adverse effects on the reproductive system in both male and female. Common effects seen in males include: reduced libido, abnormal spermatogenesis (reduced

motility and number), chromosomal damage, infertility, abnormal prostatic function and changes in serum testosterone (Gagan *et al.,* 2012).Low doses of lead were found to significantly reduce the number of sperms within the epididymis of mice, while high doses reduced both the sperm count and percentage of motile sperms and led to an increased percentage of epididymal abnormal sperms (Ab Latif *et al.,* 2015). Some authors have suggested that a delay in the development of the sex specific pituitary growth hormone secretion pattern would possibly lead to growth effects of lead rather than to a persistent defect in development (Ab Latif *et al.,* 2015).

The problems with the reproductivity of females due to lead exposure are more severe. Females are more susceptible to infertility, miscarriage, premature membrane rupture, pre-eclampsia, pregnancy hypertension and premature delivery (Flora *et al.*, 2011). Additionally, prenatal exposure to lead has been associated with toxic effects on the fetus. These include reduced gestational age, birth weight, and adversely delayed cognitive development (Saleh *et al.*, 2009). Blood lead levels in mothers and infants are usually similar as the lead present in mother blood passes into the foetus through the placenta and also through breast milk (Dart *et al.,* 2004). As

bones store the highest content of lead, if metabolic changes mobilise the lead from bones into the blood due to pregnancy, the lead toxicity risks increases. However increased calcium intake during pregnancy can help mitigate this occurrence (Grant, 2009).

2.2.7.4Effect of lead on the kidney

Lead is absorbed by the proximal tubular cells of the renal tubules, where it binds to specific lead-binding proteins. Lead can cause acute and chronic nephropathies. In kidneys, lead causes proximal tubular injury with a characteristic pathology of proximal tubule nuclear inclusion bodies that progress to tubulo-interstitial disease and fibrosis. They do not secrete

proteins in urine, but generates an abnormal excretion of glucose, phosphates and amino acids, a combination known as Fanconi's syndrome(Rastogi, 2008). On the other hand, chronic lead nephropathy is much more severe and causes irrevocable morphological and functional changes, such as diminished renal clearance, tubular reabsorption and glomerular filtration rate and tubulointerstitial changes accompanied by hypertension, hyperuricemia and renal breakdown (Rastogi, 2008; Gonick, 2008). Lead accumulates in the kidney mitochondria and causes both structural and functional alterations. These effects include mitochondrial swelling and inhibition of respiratory chain function and oxidative phosphorylation for ATP production. Consequently, energy-dependent processes, including tubular transport, are impaired (Alessia*et al.,* 2015).

2.2.7.5Effect of lead on the bone

It is well accepted that lead has effect on bone metabolism. It accumulates in human body primarily in the bones (Silbergeld *et al.,* 1993; Renner, 2010). Lead is deposited in two bone compartments: the exchangeable lead is present at the surface of bone and the non-exchangeable lead is located deeply in the cortical bone. Lead can enter into plasma at ease from the exchangeable pool but can leave the non-exchangeable pool and move to the surface only when bone is actively being re-absorbed (Patrick, 2006). In adults, 85–95% of the lead is stored in bones, in contrast to 70% in children, resulting in higher concentration of lead in soft tissues in children. The storage and the mobilization of lead in bones depends on different physiological and pathological states. These conditions include endocrine status, age, osteoporosis, and maternal age during pregnancy and lactation (Silbergeld, 1991).

2.2.7.6Effect of lead on the nervous system

The central and peripheral nervous systems (CNS and PNS, respectively) are the most sensitive targets for lead induced toxicity (Cleveland *et al.,* 2008). The effects on the

peripheral nervous system are more pronounced in adults while the central nervous system is more prominently affected in children (Bellinger, 2004; Brent, 2006). Lead exposure affects synapses formation in the cerebral cortex of children and interferes with the development of neurochemicals, including neurotransmitters, and organisation of ion channels (Ab Latif *et al.,* 2015). Blood lead levels below 10 μg/dL were reported to be associated with lower IQ and behaviour problems such as aggression, in proportion with the given blood lead level (Guidotti and Ragain, 2007). Increased blood lead levels are also associated with a decrease in cognitive performance and with other psychiatric conditions like depression and anxiety (Jacobs *et al.,* 2002). Lead-induced pathological changes also include segmental demyelination and axonal degeneration.

The brain of adults exposed to increased lead levels during their childhood showed a decreased volume, especially in the prefrontal cortex on MRI (Cleveland *et al.,* 2008). Studies have reported increase in accumulation of lead in the brain after exposure. However there is uncertainty onthe mechanism by which lead (Pb) gets into the brain. One potential mechanism is the disruption of the blood-brain barrier (BBB) by Pb. Although some investigators have reported no effect of Pb on BBB (Michaelson and Bradbury, 1982; Moorhouse *et al.,* 1988), while others have reported significant disruption of BBB by Pb (Terry, 2009; Rai *et al.,* 2010). Lead may have been able to cross the BBB by passing through the endothelial cells at the blood brain barrier because it can substitute for calcium ions and be taken up by calcium-ATPase pumps. It is also possible that Pb might have been transported into the brain by the divalent metal ion transporter 1 (DMT1), which is localized in astrocytes and neurons in the normal brain (Ong *et al.,* 2006; Wang *et al.,* 2011).

Rahman *et al.,* (2012) reported a significant accumulation of lead in the brain of Wistar rat pups that were exposed to 0.2% lead acetate via their dams' drinking water from PND 1 to

21. Du *et al.,* (2015) also reported significantly higher level of lead accumulation in Sprague–Dawley rats exposed to Pb from parturition to PND 30. The increase in accumulation of lead in the brain could also be because the gastrointestinal absorption of metal cations including Pb is much greater during lactation and pre-puberty periods because of (i) nutritional needs of young animals to grow and (ii) the involvement of the intestinal calcium-binding proteins (CaBP) in the transport of Pb, which make them more vulnerable to the deleterious effects of Pb poisoning (Alya *et al.*, 2015).

Once in the brain, lead-induced damage in the prefrontal cerebral cortex, hippocampus, and cerebellum can lead to a variety of neurological disorders, such as brain damage, mental retardation, behavioural problems, nerve damage, and possibly Alzheimer's disease, Parkinson's disease, and schizophrenia. Lead toxicity leads to encephalopathy with a progressive degeneration of certain regions of brain, the major symptoms including dullness, loss of memory, hallucinations, headache, poor attention span, and irritability (Ab Latif *et al.,* 2015).

Lead has negative effect especially on the developing nervous system of the foetuses and young children, which absorbs a higher fraction of this metal. Children with blood lead concentration greater than 10μg/dL are at higher risk for developmental disabilities (Ab Latif *et al.,* 2015). The effect of lead on children´s cognitive abilities takes place at very low levels (Park *et al.,* 2008; Xu *et al.,* 2009; Sanders *et al.,* 2009). There is apparently no lower threshold to the dose-response relationship below which lead exposure is treated as safe because blood lead levels lesser than 3 μg/dL were found to be associated with reduced academic performance (Needlemann *et al.,* 1990; Canfield *et al.,* 2003; Meyer *et al.,* 2003; Bellinger, 2008).

Needleman *et al.,* (2004) reported that countries with the highest lead levels in the air deviate normal behaviour and turn to become aggressive and violent, thus for example the highest murder rates were found in countries with high levels of lead in the air. This was supported by Park *et al.,* (2008) who also reported a correlation between prenatal and early childhood lead exposure with violent crimes in adulthood. With increased lead exposure in children, an increase in neuropsychiatric disorders like attention deficit hyperactivity disorder and antisocial behaviour were found (Sanders *et al.,* 2009).

The activity of the hippocampus (a part of the brain involved in learning and memory) is distorted particularly in children by exposure to lead. Lead damages the cells within the hippocampus hence causing memory and learning deficit. Structural damage such as irregular nuclei and denaturation of myelin were also reported in rats (Ab Latif *et al.,* 2005). Lead also interferes with the release of neurotransmitters (Dart *et al.,* 2004; Needlemann, 2004). This interference leads to the disruption of communication between cells. Lead usually interferes with the neurotransmitter glutamate which is important for many functions, like learning and memory. It operates by blocking N methyl –D- aspartate receptors (NMDAR) which is thought to be the main target of lead toxicity. One study found that in addition to inhibition of the NMDA receptor, lead exposure also decreased the amount of gene for this receptor in part of the brain (Kosnett, 2005).

2.2.8 Prevention and treatment of lead poisoning

Being unnecessary for the human body, the prescribed limit for drinking water set by WHO is 0.01 mg/L (10 ug/L) (Karrari *et al.,*2012). On the recommendations of the Advisory Committee on Childhood Lead Poisoning Prevention (ACCLPP), the term blood lead level (BLL) which was prevalent method for assessing Pb exposure and, as such, its toxicity has now been replaced by “BLL reference level”. Over the years, BLL reference level set by the

Center for Disease Control (CDC) has changed from 60 ug/dL in 1960s to 5 ug/dL for children in the age group of 1–5 years (ACCLPP, 2000). Acceptable BLL reference level for the working population ranges between 30 and 49.9 ug/dL (Abdollahi *et al.*,1996).

However, BLL reference level of <49 ug/dL was associated with impaired cognitive, behavioural, and motor development in children and, along with this, hypertension, nephropathy, and impaired fertility in adults (Nelson *et al.,* 2011). Adults with BLL less than 70 ug/dL generally do not require chelation therapy. However, chelation therapy with succimer is advisable as a treatment option for children with BLL between 45 and 69 ug/dL (Rogan *et al.,* 2001). The best approach to prevent lead toxicity is to avoid exposure to lead (Rossi, 2008).

It is recommended that children´s hands are frequently washed and also to increase their intake of calcium and iron. It is also suggested that children should be discouraged from putting their hands, which may be contaminated, in their mouth regularly. Frequent vacuuming and eradication of the use of lead containing objects like blinds and jewellery in the house can also help to prevent exposures. House pipes containing lead or plumbing solder fitted in old houses should be replaced to avoid lead contamination through drinking water. It is believed that hot water contains higher lead levels than does cold water, so it is recommended that for household uses cold water should be preferred to hot water (Baselt, 2008).

Lead poisoning is generally treated by using chelating salt disodium calcium edentate, which is the calcium chelate of the disodium salt of ethylene-diamine-tetracetic acid (EDTA), dimercaprol and succimer (Park *et al.,* 2008). Such chelating agents have a great affinity to the removing agent. The chelating agent for lead has a greater affinity to lead than calcium and so the lead chelate is formed by exchange. This is then excreted in urine, leaving behind

harmless calcium (Ab Latif *et al.,* 2015). Blood lead levels were shown to be lowered by treatment with succimer used as chelation therapy in children exposed to lead to improve their neuropsychological development. And yet, though succimer was observed to help in reducing blood lead levels, it failed in improving the scores of cognition tests (Rogan *et al.,* 2001).

There are a number of antioxidants which are believed to act against toxicity of chemicals like lead and its related compounds. Also a new technique called nano-encapsulation of antioxidants may provide improved biodistribution and bioavailability of poorly soluble therapeutics through solubilisation (Gagan *et al.,* 2012). While recognizing that even a small amount of it is harmful, it appears that reducing its toxicity in humans with use of chelating agents is not possible. Although exposure mitigation strategies imposed by government or through public health interventions have led to a significant decline in the symptomatic poisoning of the human population, childhood lead poisoning is still regarded as an important public health concern worldwide (Arif *et al.,* 2015).

2.3 Hippocampus

The hippocampusis a part of the limbic system that lies caudal to and intimately connected with the amygdala. It is a bilaminar gray-matter structure that has the shape of a curved tube, which resembles a seahorse, a ram's horn or a banana and forms the floor of the inferior temporal horn of the lateral ventricle (Figure 2.4) (Campbell and MacQueen, 2004). It extends from the anterior margin of the ventricular horn to the splenium of the corpus callosum (Amaral and Lavenex, 2006). It is the posterior part of limbic system while the frontal part is amygdala. Like the cerebral cortex, with which it is closely associated, it is a paired structure, with mirror-image halves in both sides of the brain that plays important roles in long-term memory and spatial navigation(Campbell and MacQueen, 2004). In humans and

other primates, the hippocampus is located inside the medial temporal lobe, beneath the cortical surface. Though lies sub-cortically, it is not totally considered to be a sub-cortical structure.

In adult humans, the volume of the hippocampus on each side of the brain is about 3-3.5 cm^3 as compared to 320-420 cm^3 for the volume of the neocortex. Thus, hippocampus is 100 times smaller in volume compared to cerebral cortex (Gilbert and Brushfield, 2009). Psychologists and neuroscientists generally agree that the hippocampus plays an important role in the formation of new memories (episodic or autobiographical memory) (Squire and Schacter, 2002). Part of this function is hippocampal involvement in the detection of new events, places and stimuli (VanElzakker *et al.*, 2008). Some researchers regard the hippocampus as part of a larger medial temporal lobe memory system responsible for general declarative memory (memories that can be explicitly verbalized—these would include, for example, memory for facts in addition to episodic memory) (Squire and Schacter, 2002).

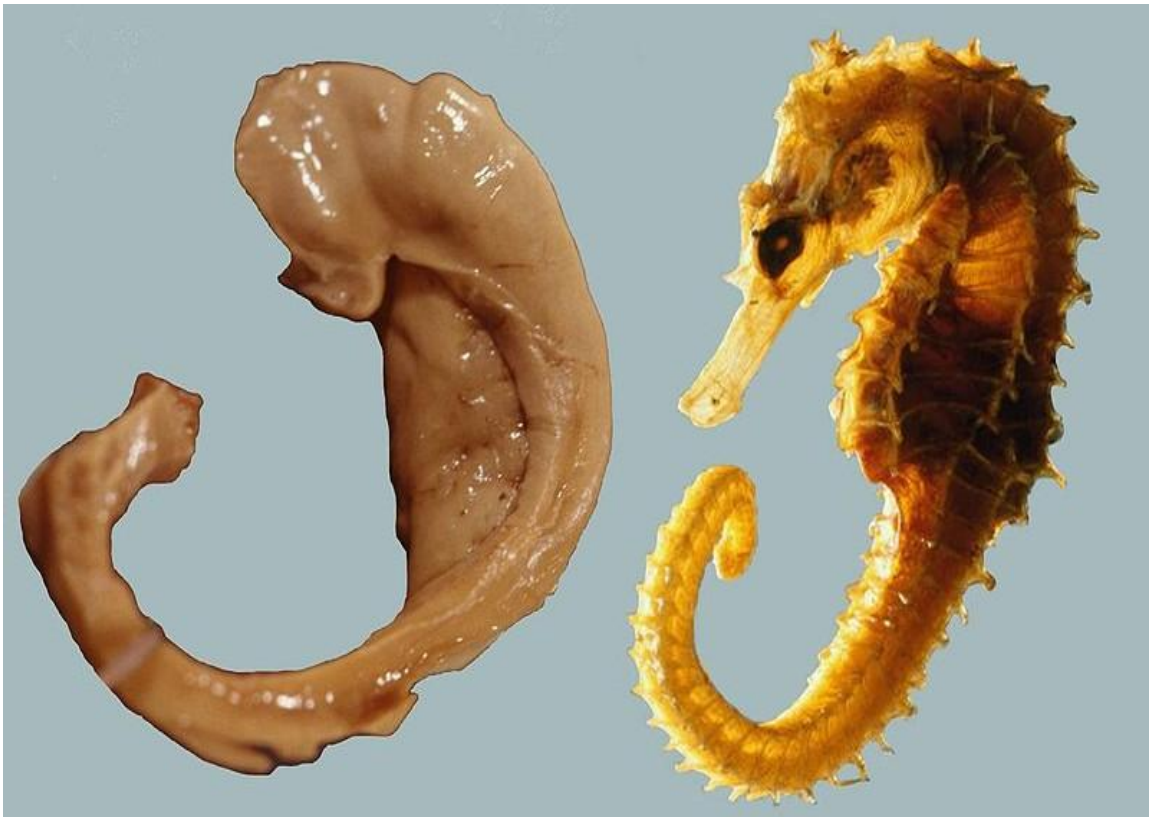


Figure 2.4: The sea horse shape of the hippocampus
(Campbell and MacQueen, 2004)

The hippocampus also encodes emotional context from the amygdala. This is partly why returning to a location where an emotional event occurred may evoke that emotion. There is a deep emotional connection between episodic memories and places (Gluck *et al.*, 2014). Due to bilateral symmetry the brain has a hippocampus in each cerebral hemisphere. If damage to

the hippocampus occurs in only one hemisphere, leaving the structure intact in the other hemisphere, the brain can retain near-normal memory functioning. Severe damage to the hippocampi in both hemispheres results in profound difficulties in forming new memories (anterograde amnesia) and often also affects memories formed before the damage occurred (retrograde amnesia) (Di Gennaro *et al.*, 2006).

2.3.1 History of hippocampus nomenclature

The ridge running along the floor of the temporal horn of the lateral ventricle was the earliest description of the hippocampus and comes from the Venetian anatomist Julius Caesar Aranzi (1587), who initially likened it to a silkworm, and then a seahorse. Duvernoy a German scientist the first to illustrate the structure in 1729, also wavered between seahorse and silkworm. Ram's horn was proposed by the Danish anatomist Jacob Winslow in 1732 (figure 2.4); and a decade later his fellow Parisian, the surgeon de Garengeot, used "cornu Ammonis" - horn of (the ancient Egyptian god) Amun. Today, the structure is called the hippocampus (Duvernoy, 2005). De Garengeot's "cornu Ammonis" survives in the names of the hippocampal subfields CA1– CA4 (Wechsler, *et al.*, 2004).

2.3.2Development of the hippocampus

The hippocampus begins to develop as a narrow strip of cortical tissue trapped along the medial wall of the hemisphere. As it expands, it finds nowhere to go except into the ventricle. It first pushes laterally and then doubles back underneath itself. The U-shape of the adult fascia dentata has been suggested to be due to the pressure of the hippocampus proper invaginating into the granule-cell layer during its development and astrocytes (spongioblasts) are concentrated in a layer adjacent to the ventricle called the matrix lamina. Here, new neuroblasts are continually formed by cell division. After their final division the immature

neurons migrate towards the pial surface where they congregate in the cortical plate(Stensaas, 1967).

As in all cortical tissue, with the exception of the fascia dentata, the formation of the cortical plate takes place in an inside/out manner; newly formed neurons of the hippocampus proper move up through the older neurons of the cortical plate and come to rest at the edge facing the pial surface. Stensaas, (1967) suggested that the migrating neuroblasts encounter a condition at this level which discourages movement and promotes the growth of dendrites. As development proceeds, the number of neurons in the cortical plate rapidly increases and the hippocampus begins to take on its characteristic enfolded shape. During this period the future fascia dentata is represented by a pool of germinal cells gathered above the end of the cortical plate towards the fimbria. The development of the fascia dentata lags well behind that of the rest of the hippocampus, and indeed behind the rest of the brain in general, continuing postnatally in some species (Albani *et al.,* 2014).

Hine and Das, (1974) showed that the vast majority of the pyramidal cell of the hippocampus proper are formed prenatally in the mouse and rat. On the other hand approximately 85 per cent of the dentate granules originate after birth, as do a fair proportion of the interneurons in all parts of the hippocampus. Of the 85 per cent of granule cells formed postnatally 25 per cent originate during the first four days after birth and 20per cent more in the next four days (Bayer and Altman, 1975). The formation of synapses between the perforant path axons from the entorhinal cortex and the dendrites of the granule cells takes place in step with cell formation; the biggest surge in synaptic formation occurs in the period between postnatal days 4 and 11 when the number of synapses in the exposed blade doubles every day and the synaptic density increases 20 times.

Mellgren, (1973) described that aside from the postnatal development of fascia dentata cells there is also postnatal development of the cholinergic input systems to the hippocampus. This

may account for the fact that the two separate theta patterns develop at different times. The CA1 region seems responsible for the triggering of exploration, and the time of onset of theta activity from CA1 agrees nicely with the onset of exploration in neonates(Albani *et al.*, 2014). This suggests a possible function of postnatal development; the delayed maturation of the hippocampus could have the negative effect of delaying the onset of exploration. During the period when the animal is still dependent upon the mother it would be dysfunctional for it to be continually leaving the nest to explore its environment. Spontaneous alternation, which is a reliable measure of exploration, develops around day 28 in rats (Albani *et al.*, 2014).

2.3.3 Structure of the hippocampus

The hippocampus consists of ventral and dorsal portions, both of which share similar composition but are parts of different neural circuits (Moser and Moser, 1998). In the axial and sagittal plane, it can be divided into three parts: (1) the head or anterior expanded segment; (2) the body or intermediate segment; and (3) the tail or posterior thin curved segment. This general layout holds across the full range of mammalian species, from hedgehog to human, although the details vary (Winson, 1978). In the rat, the two hippocampi resemble a pair of bananas, joined at the stems. In human or monkey brains, the portion of the hippocampus down at the bottom, near the base of the temporal lobe, is much broader than the part at the top. Together with the dentate gyrus (DG) and parahippocampal regions, including the subiculum, presubiculum, parasubiculum, and the entorhinal cortex (EC), the hippocampus is thought to play a key role in memory and navigation (Deshmukh and James, 2012).

White matter fibres from the hippocampus accumulate on its superior surface to form the alveus. While white matter fibres from the alveus then gather medially into thickened bundles as the fimbria, which are continuous posteriorly with the fornix (Duvernoy, 2005; Dekeyzer

et al., 2017). The hippocampus is surrounded by several fissures which are collectively referred to as the perihippocampal fissures. The transverse fissure of Bichat is the lateral extension of the ambient cistern which separates the thalamus superiorly from the parahippocampal gyrus inferiorly. The superolateral extension of the transverse fissure is the choroidal fissure. The inferolateral extension of the transverse fissure is the hippocampal fissure, which extends between the dentate gyrus and the subiculum and is often obliterated and not visible on MRI (Dekeyzer *et al.,* 2017).Furthermore, the hippocampus consists of two interlocking gray matter folds, the cornu ammonis (or hippocampus proper) and the dentate gyrus. Both of these parts are separated by hippocampal sulcus and curve into each other(Dekeyzer *et al.,* 2017).

2.3.4 Hippocampal formation

From the dentate gyrus inward along the S-curve of the hippocampus are a series of narrow zones. The dentate gyrus (DG), is actually a separate structure with tightly packed layer of small granule cells wrapped around the end of the hippocampus proper. It forms a pointed wedge in some cross-sections, a semicircle in others (Amaral *et al.,* 2007). Afterward come a series of Cornu Ammonis (CA) areas: first CA4 (which underlies the dentate gyrus), then CA3, then a very small zone called CA2, then CA1. The CA areas are all filled with densely packed pyramidal cells similar to those found in the neocortex. After CA1 comes an area called the subiculum(Figure 2.5) (Amaral *et al.,* 2007).Immediately after the subiculum is a pair of ill-defined areas called the presubiculum and parasubiculum, then a transition to the cortex proper (mostly the entorhinal area of the cortex). Most anatomists use the term "hippocampus proper" to refer to the four CA fields, and hippocampal formation to refer to the entorhinal cortex, dentate gyrus, hippocampus proper and subiculum (Amaral and Lavenex, 2006).

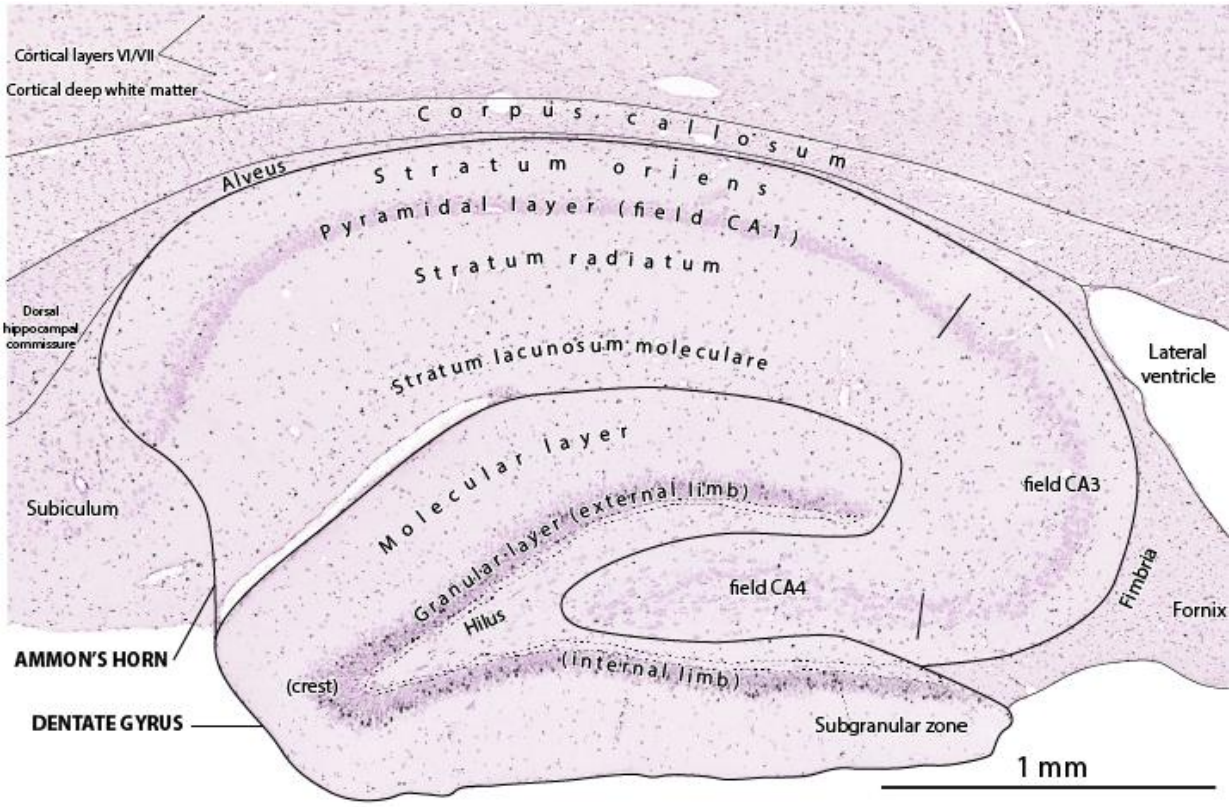


Figure 2.5: Subdivision of the hippocampal formation
(Dekeyzer *et al.*, 2017).

2.3.4.1Entorhinal cortex

The entorhinal cortex is divided into medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC) areas. It is the entrance for majority of the cortical input to the hippocampus and also receives output from the CA1 layer via the subiculum(Wible, 2013). It has six well-defined layers. The neurons in layers II and III send major inputs to the hippocampus, dentate gyrus, and the subiculum, while the neurons in layers V and VI receive feedback from CA1 and subiculum (Figure 2.6)(Deshmukh and James, 2012).

The entorhinal cortex has its main reciprocal connections with the perirhinal and parahippocampal cortices. Hence, the hippocampus communicates with a widespread of cortical areas through the entorhinal, perirhinal and parahippocampal cortices (Lavenex *et al.,* 2002; Wible, 2013). The hippocampus also contains fiber pathways that run longitudinally throughout its extent. This would allow for the excitation between disparate portions of the hippocampal formation. The entorhinal cortex extends from the amygdala (anteriorly) to approximately 10 mm posterior to the most anterior aspect of the hippocampal fissure (Sloviter and Lomo, 2012).

2.3.4.2Dentate gyrus

The dentate gyrus is part of the hippocampal formation believed to contribute to the formation of new episodic memories, spontaneous exploration of novel environments, and other functions (Amaral *et al.,* 2007; Saab *et al.,* 2009). It is one of the few brain structures currently known to have significant rates of adult neurogenesis in many species of mammals, from rodents to primates (Saab *et al.,* 2009). The dentate gyrus (DG) consists of three distinct layers: molecular, granular, and polymorphic layers(Wible, 2013). The granule cell layer are made up of neurons called granule cells. This cells form a horizontal U or Vshaped layer

(Figure 2.5), with dendrites that extend into the molecular layer and receive excitatory inputs from layer II of the EC neurons via the perforant path. The input to the dentate gyrus is made up of signals principally from layer II of the entorhinal cortex, with no direct inputs from other cortical structures (Figure 2.6)(Wible, 2013).

In a coronal section through the septal hippocampus, the two arms of this U or V are referred to as the upper and the lower blades, with the upper blade being more dorsal of the two (Figure 2.5). The deeper layer of the DG, called the polymorphic cell layer (or hilus), has a variety of neurons (Figure 2.5). Mossy fibres (the axons of granule cells) give rise to collaterals that synapse onto some of the neurons in the polymorphic cell layer, including the mossy cells and on the dendrites of CA3 pyramidal neurons. Mossy cells are glutamatergic neurons that project back onto the granule cells and inhibitory interneurons along the septotemporal axis (Deshmukh and James, 2012). Thus, the granule cells and mossy cells form both disynaptic and trisynaptic feedback loops.

2.3.4.3Cornu ammonis (CA)

The Cornu Ammonis (CA) fields of the hippocampus consist of pyramidal cells that are divided into four parts, the so-called Sommer's sectors CA1, CA2, CA3, and CA4 (Figure 2.5).

Cornu Ammonis 1 (CA1):

It is the first region in the hippocampal circuit with a major output pathway to layer V of the entorhinal cortex (EC) and also to the subiculum. CA1 receives inputs from the CA3 via the proximal-distal organization of the Schaffer collaterals. It also receives inputs from the EC that correspond to the proximal-distal organization of the Schaffer collaterals(Naber*et al.,* 2001). Unlike CA3 and dentate gyrus(DG), where the inputs from the medial entorhinal cortex (MEC) and the lateral entorhinal cortex (LEC) are distributed equally along the

transverse axis, MEC layer III neurons project to proximal CA1 while LEC layer III neurons project to distal CA1 (Figure 2.6)(Deshmukh and James, 2012). Thus, proximal CA1 receives input principally from the MEC and from distal CA3, whereas distal CA1 receives input primarily from LEC and proximal CA3(Hargreaves *et al.,* 2005). Because LEC and MEC are thought to convey fundamentally different types of information to the hippocampus, (Deshmukh *et al.,* 2010; Deshmukh and Knierim, 2011) and proximal and distal CA3 are also thought to perform different computational processing, it is likely that the proximal and distal regions of CA1 are functionally distinct (Hunsaker *et al.,* 2008; Henriksen *et al.,* 2010).

Cornu Ammonis 2 (CA2):

It is a small region located between CA1 and CA3. Best defined as the strip of cells that receive perforant path (PP) input from EC layer II but do not receive mossy fiber connections from dentate gyrus. Its pyramidal cells are more like those in CA3 than those in CA1. It is often ignored due to its small size.CA2 just like the CA1 has four layers except the lucidum stratum and it is the most resistant subfield to injury secondary to seizures, hypoxia, and other insults (Deshmukh and Knierim, 2011).

Cornu Ammonis 3 (CA3):

It is composed of an extensive population of pyramidal neurons. The pyramidal neurons of the CA3 region forms a well-defined cell layer starting in between the upper and lower blades of the dentate gyrus. It receives inputs from the mossy fibers of the dentate gyrus, the medial septum, diagonal band of Broca, the contralateral hippocampus (via commisures) as well as from the neurons of layer II of the entorhinal cortex via the perforant path (Figure 2.6)(Deshmukh *et al.,* 2010). CA3 pyramidal cells make two major projections within the hippocampus. The recurrent collateral system comprises CA3 axons that make synapses onto other CA3 pyramidal cells. Collaterals of the same axons that form the recurrent collateral

system also project forward to the CA1 region in a pathway called the Schaffer collateral system (Deshmukh and James, 2012).

The Schaffer collaterals show a structural organization in their projections along the transverse axis of CA1. To understand these projection patterns, it is necessary to acquaint with the anatomical terminology with which the cells along the CA layer are described in terms of their proximity to the DG. Cells that are closer to the DG are called proximal, whereas cells that are farther away are called distal. Distal CA3 pyramidal cells project preferentially to proximal CA1 cells, at levels more temporal to the cells of origin, while proximal CA3 neurons project more to distal CA1 cells at levels more septal to the cells to origin (Figure 2.6). The CA3 has the following cell layers known as strata: lacunosum-moleculare, radiatum, lucidum, pyramidal, and oriens (Figure 2.5) (Deshmukh and James, 2012).

Cornu Ammonis 4 (CA4):

It is often called the hilus or hilar region if considered as a part of the dentate gyrus. Because CA4 pyramidal neurons do not have the typical morphology of other CA pyramidal cells, it is considered by some as part of the dentate gyrus. Unlike the pyramidal neurons in CA1 and CA3, the neurons here contain mossy cells that primarily receive inputs from the granule cells in the dentate gyrus in the form of mossy fibres. They also receive a small number of connections from pyramidal cells in CA3. They, in turn, project back into the dentate gyrus at distant septotemporal levels (Deshmukh and James, 2012).

2.3.4.4Subiculum

The subiculum is the medial and superior edge of the parahippocampal gyrus and its site of union with the cornu ammonis (Duvernoy, 2005). The subiculum undergoes an unusual and striking increase in myelination during late adolescence. The subiculum also shows the same

type of segregation along the proximal distal axis as the CA1 region, as lateral entorhinal cortex (LEC) layer III projects to proximal subiculum and medial entorhinal cortex (MEC) layer III projects to distal subiculum (Deshmukh and James, 2012). The projections of CA1 and subiculum backward to layers V and VI of entorhinal cortex (EC), in turn, maintain this structure, with proximal CA1 and distal subiculum projecting to medial entorhinal cortex (MEC) and distal CA1 and proximal subiculum projecting to lateral entorhinal(LEC) (Figure 2.6). This anatomical segregation implies that there are two distinct processing streams through the hippocampal formation, one that is associated with MEC inputs (thought to relay spatial information based on path integration) and the other associated with LEC inputs (thought to relay information about objects and external landmarks) (Deshmukh and James, 2012).

2.3.5 Information flow in the hippocampal formation

Andersen *et al.,* (1971) using the anatomical and physiological evidence available at the time formulated the lamellar hypothesis, which suggested that the synaptic connections between the subregions of the hippocampal formation are limited to a thin slice (lamella) orthogonal to the septotemporal axis. Thus, a transverse strip of hippocampus was thought to possess the entire 'trisynaptic pathway' (Andersen *et al.,* 1971) which comprises the EC axons and terminals connecting to DG, DG connections to CA3, and CA3 connections to CA1. According to this view, the hippocampus was organized along the septotemporal axis as a stack of such lamellae, each operating as a mostly independent functional unit (Witterand Amaral, 2004).

Studies have showed that, apart from the mossy fibers connecting DG to CA3, all pathways in the hippocampal formation are more divergent along the septotemporal axis than suggested by the lamellar hypothesis (Amaral and Witter, 1989). Furthermore, the synaptic connectivity

within the hippocampal formation is much more complex than the classic 'trisynaptic pathway,' with a number of parallel input pathways and feedback pathways.

The major pathways of signal flow through the hippocampus combine to form a loop. Most external input derives from the adjoining entorhinal cortex, via the axons of the perforant path. These axons arise from layer II of the entorhinal cortex (EC), and terminate in the dentate gyrus and CA3. These axons also project from layer III of the EC directly to CA1, often referred to as the temporoammonic or TA-CA1 pathway. The granule cells of the DG send their axons (called mossy fibers) to CA3. Pyramidal cells of CA3 send their axons to CA1. Pyramidal cells of CA1 send their axons to the subiculum and deep layers of the EC. Subicular neurons send their axons mainly to the EC (Figure 2.6) (Deshmukh and James, 2012).Perforant path input from EC layer II enters the dentate gyrus and is relayed to region CA3 (and to mossy cells, located in the hilus of the dentate gyrus, which then send information to distant portions of the dentate gyrus where the cycle is repeated). Region CA3 combines this input with signals from EC layer II and sends extensive connections within the region and also sends connections to strata radiatum and oriens of ipsilateral and contralateral CA1 regions through a set of fibers called the Schaffer collaterals, and commissural pathway, respectively (Laurberg, 1979). So that the CA1 region of the hippocampus receives input from the CA3 subfield, EC layer III and the nucleus reuniens of the thalamus (which project only to the terminal apical dendritic tufts in the stratum lacunosum-moleculare). In turn, CA1 projects to the subiculum as well as sending information along the aforementioned output paths of the hippocampus.

The subiculum is the final stage in the pathway, combining information from the CA1 projection and EC layer III to also send information along the output pathways of the hippocampus (Figure 2.6) (Heath and Harper, 1974). The hippocampus also receives a

number of subcortical inputs from the amygdala (specifically the anterior amygdaloid area, the basolateral nucleus, and the periamygdaloid cortex), the medial septum and the diagonal band of Broca, the claustrum, the substantia innominata and the basal nucleus of Meynert, the thalamus (including the anterior nuclear complex, the lateral preoptic and lateral hypothalamic areas, the supramammillary and retromammillary regions, the ventral tegmental area.(Heath and Harper, 1974).

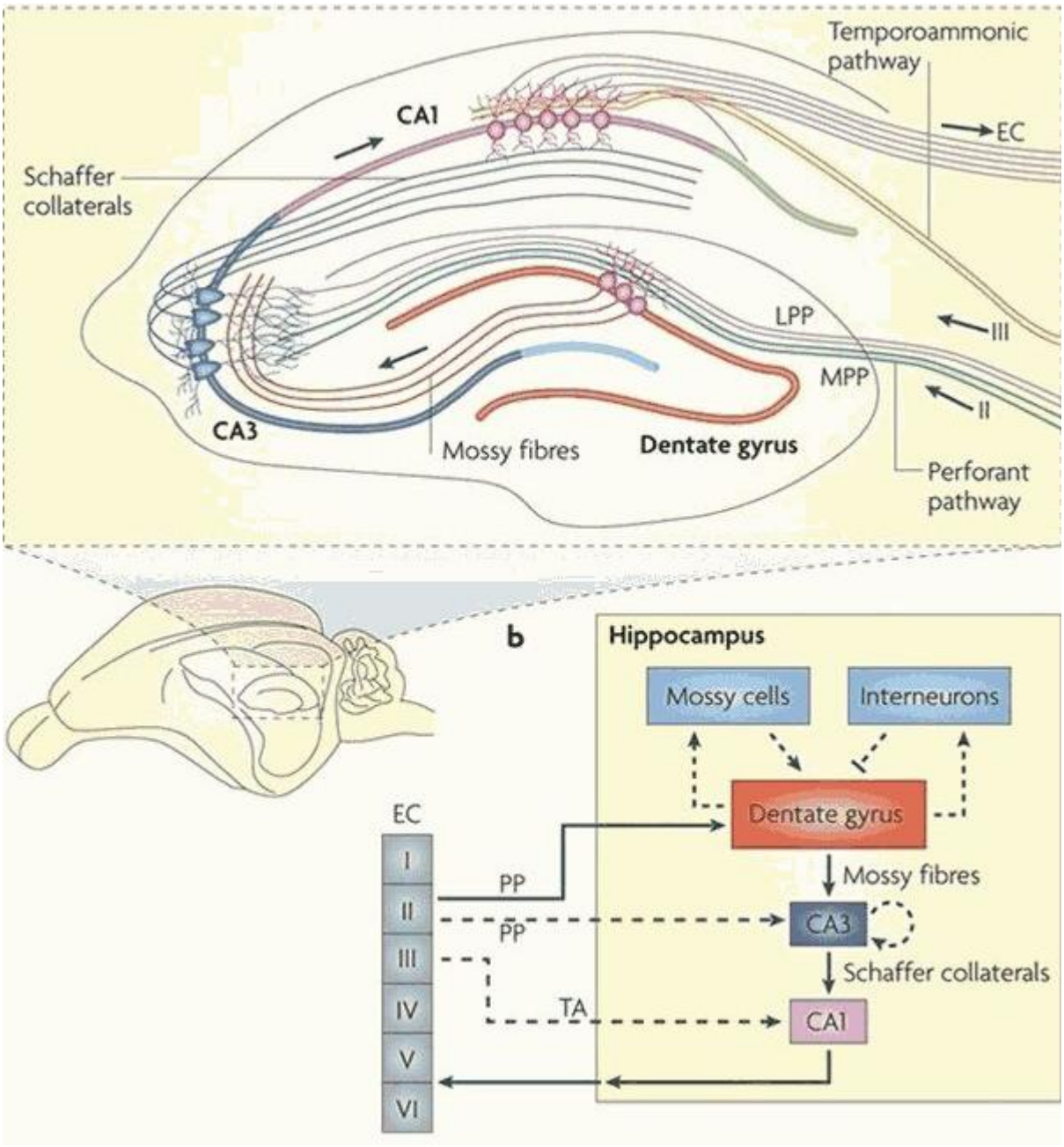


Figure2.6: Information flow in the hippocampal formation
(Deshmukh and James, 2012)

2.3.6 Functional implications of anatomical differences between hippocampal sub regions

The numbers of neurons in different regions and the connectivity patterns between regions have led to hypotheses about the nature of information processing in different regions. About 300,000 neurons from EC project to approximately 1,000,000 granule cells in DG. In comparison, CA3 has approximately 250,000 pyramidal neurons and CA1 has 400,000 (Witter and Amaral, 2004). The relatively small number of EC neurons projecting onto a larger number of dentate gyrus granule cells has led to the hypothesis that DG performs pattern separation, by creating sparse, nonoverlapping representations from entorhinal cortex input.

In contrast, CA3 pyramidal neurons, with their extensive recurrent connectivity, are thought to perform pattern completion from DG and/or EC inputs, allowing recall of memories from degraded or incomplete inputs (McNaughton and Morris, 1987; Deshmukh and James, 2012). Recurrent connectivity within CA3 may also play a role in one-trial learning and sequence learning (Knierim *et al.,* 2006). Finally, CA1 pyramidal neurons receive inputs from CA3 as well as EC, and thus can compare the recalled information (Deshmukh and James, 2012).The hippocampus has been one of the most extensively studied, and atrophy of this region has clinical consequence (Dhikav, 2006; Dhikav and Anand, 2007). It is the earliest and most severely affected structure in several neuropsychiatric disorders such as Alzheimer's disease (AD), epilepsy (Dhikav and Anand, 2007; Dhikav *et al.,* 2009; Frisoni *et al.,* 2010; Dhikav and Anand, 2011).

The physiology of the hippocampus is unique and bestows the region with a high level of plasticity that is important for learning and memory. Memory can be described as the accurate storage and recall of information by the brain. Changes in synaptic efficacy are

thought to be a major mechanism of information storage, ever since Cajal hypothesized that neurons communicate with each other at the synapses (Cajal, 1995). This came to be called long-term potentiation (LTP). LTP was later demonstrated in the dentate gyrus (DG) mossy fiber to CA3 pyramidal cell synapses and in the CA3 Schaffer collateral to CA1 pyramidal cell synapses in hippocampal transverse slices as well as in numerous brain areas outside the hippocampal formation (Deshmukh and James, 2012).

The NMDA (N-methyl D-aspartate) subtype of glutamate receptor, is a dual voltage- and ligand-(glutamate) gated Ca^{2+}channel. It is critical for the induction of LTP at many synapses, including the perforant path-DG synapse and the Schaffer collateral-CA1 synapse (Deshmukh and James, 2012). At the resting membrane potential, the pore of the NMDA receptor is blocked by Mg^{2+} ions, such that release of glutamate by a presynaptic cell is insufficient, by itself, to open the channel. Rather, the postsynaptic cell have to be depolarized in order to electrostatically repel the Mg^{2+}ion from the channel, at the same time that glutamate is bound to the receptor, in order for the channel to open (Deshmukh and James, 2012).

This characteristic gives the NMDA receptor the properties of a coincidence detector of both presynaptic (glutamate release) and postsynaptic (depolarization) activity. The hippocampus and, in particular, one subfield, the CA1 layer (output layer), has the highest concentration of N-Methyl-D-aspartate (NMDA) receptors in the brain (Lee *et al.,* 2004). Neurogenesis is a form of plasticity distinct from synaptic plasticity, it involves the formation of new neurons from precursor cells. The DG is one of only two brain areas in mammals that show neurogenesis throughout the adult life span, contrary to the long-held belief that no new neurons could be generated in the adult brain (Deshmukh and James, 2012). Adult

neurogenesis in DG is thought to play a role in learning and memory, as well as pattern separation. (Deng *et al.,* 2010).

Single unit (neuronal) activity has been shown to be related to a wide variety of stimuli within various tasks or contexts in humans and other animals, including words, pictures locations, odours and sounds (Fried *et al.,* 2002; Eichenbaum, 2004; Quiroga *et al.,*2005; Quiroga *et al.,* 2008). Unit recording studies also show that although hippocampal system function may not be necessary for the maintenance of short-term memory or working memory, it is active during these types of memory tasks. In fact, if the contents of working memory cannot be actively rehearsed or if this process is interrupted, then the hippocampus is needed to hold the memory, even at short time periods (Hannula *et al.,* 2006).

2.4 Microglia

Microgliaare glial cells found throughout the central nervous system (CNS) (Ginhoux *et al.,* 2013). They are the resident macrophages of the brain and spinal cord with important roles in development, homeostasis, disease, and injury (Aguzzi *et al.,* 2013). This glia cells account for 5–20% of the total glial cell population within the central nervous system (CNS) parenchyma (Perry, 1998). The microglia is defined by two key functional features; immune defence and maintenance of CNS homeostasis. As part of the innate immune system, microglia were initially proposed as ‘‘resting’’ immune cells that only became active in response to pathological events in the brain (Kreutzberg, 1996). However, it is now known that adult microglia are highly dynamic cells, constantly sampling their environment, scanning and surveying for signals of external danger (Davalos *et al.,* 2005; Nimmerjahn *et al.,* 2005; Lehnardt, 2010), such as those from invading pathogens, or internal danger signals generated locally by damaged or dying cells (Bessis *et al.,* 2007; Hanisch and Kettenmann,

2007) in this delicate immune-privileged site that is actively protected by the brain blood barrier (BBB) (Daneman, 2012).

The microglia cells are associated with the BBB and are among the first cells to respond to threats to the brain (Midori *et al.,* 2006). They provides the first line of defence against any disease or insult and display enormous structural and functional plasticity which may include; proliferation, chemotaxis, morphological alterations, and generation of immunomodulatory molecules (Benarroch, 2013).Microglial density varies in distinct brain locations, with a higher density being evident in grey matter in rodents (Benarroch, 2013). They are also known as multi-tasking effectors and regulators in the CNS, capable of playing both pathogenic and protective roles during various phases of neurological diseases (Grubman *et al.,* 2016; Jinming, *et al.,* 2017).

During physiological conditions, as the first housekeeper of the microenvironment against pathogens, resting microglia are characterized by a small cell body and ramified morphology with multiple branches that actively and efficiently scan the surrounding environment, ensuring CNS homeostasis and impacting neural development (Michell-Robinson *et al.,* 2015). Upon stimulation, microglia withdraw their processes and rapidly convert into an amoeboid activated morphology state. They release bioactive molecules, thus contributing to active inflammatory responses and combating foreign invasion at damaged sites (Jin and Yamashita, 2016). Activated microglia are thus plastic cells that may adopt diverse phenotypes such as ramified, amoeboid and others in response to different stimuli and various micro environmental changes (Shemer *et al.,* 2015; Thompson and Tsirka, 2017).

2.4.1 Development of the microglia

Although microglia have been studied for more than 100 years, the precise origin of microglia and its cell lineage still remains highly controversial. The evident phenotypic

similarities between microglia and other macrophage populations led to acceptance of their myeloid origin. They are the only cell population in the CNS that originate outside the brain (Ginhoux and Prinz, 2015). Some researchershave argued that microglia originated from hematopoietic stem cells (HSCs). Microglia differentiate from macrophages produced by the yolk sac, the first site of haematopoiesis(Figure 2.7)(Herbomel *et al.,* 2001; Ginhoux *et al.,* 2010).

The differentiation of yolk sac macrophages into typical microglia is dependent on transcription factors like IFM regulatory factor-8 (IRF-8), Runx1 and Pu.1 and independent of transcription factor Myb which is important in controlling HSC differentiation (Ginhoux *et al.,* 2013; Prinz and Priller, 2014). During development, Pu.1 is responsible for the transition from microglial precursors to the A1 state, while IRF8 is essential for the maturation from the A1 to A2 state (Tay *et al.,* 2016). Once born, yolk sac macrophages migrate into the body of the embryo where they colonize various organs. The brain is the first organ to be colonized, even before the liver or lungs (Herbomel *et al.,* 2001). Hence the distinction between microglia and other macrophages has often been based on their tissue localization (Ginhoux *et al.,* 2010; Ransohoff and Cardona, 2010; Prinz and Mildner, 2011).

The microglia enters the embryonic brain and resides before the differentiation of other cell types. HSCs can be detected at around embryonic day 10.5 (E10.5), while F4/80+CD11b+ microglia during embryogenesis can be found as early as E8.0 and yolk sac progenitors begin to migrate to the brain at around E9.5 and grow as amoeboid microglia (Ginhoux *et al.,* 2010; Prinz and Priller, 2014; Prinz *et al.,* 2017). The presence of primitive microglial precursors in the developing brain earlier than definitive haematopoiesis indicates that microglia in fact exist independently of HSCs (Prinz and Priller, 2014). Using cutting edge genetic fate mapping tools, Ginhoux *et al.,* (2013)strengthened this concept by reporting for

the first time that microglia exclusively derive from progenitors in the yolk sac during early development, and can be constantly self-renewed throughout adult life with no input from peripheral cells in the healthy situation (Jinming, *et al.*, 2017).

Potential non-yolk sac sources such as fetal-liver progenitor cells may also give rise to microglia(Figure 2.7). A recent study showed that fetal-liver progenitor cells could contribute to the resident microglial pool only at early postnatal stages due to rapid depletion by apoptosis (Askew *et al.*, 2017). Bone marrow-derived progenitors or monocytes are also considered to be recruited for supplementing the microglial population(Figure 2.7) (Saijo *et al.*, 2013; Ginhoux *et al.*, 2013). When it comes to certain conditions such as whole body irradiation and global depletion of microglia, however, the repopulation of CNS local dying or depleted microglia by bone marrow-derived macrophages that adopt a microglia-like phenotype has also been reported (Prinz and Priller, 2014).

Also, Bone chimera studies have shown, however, that in cases of extreme infection the blood–brain barrier will weaken, and microglia will be replaced with myeloid progenitor cells and macrophages from the bone marrow(Figure 2.7). Once the infection has decreased the disconnection between peripheral and central systems is re-established and only microglia are present for the recovery and regrowth period (Gehrmann, 1996). While we continue debating the microglial lineage and origin, interestingly two independent reports claim that microglia can themselves act like pluripotent stem cells and can also transform into astrocytes, neurons and oligodendrocytes (Yokoyama *et al.*, 2004; Matsuda *et al.*, 2008) although the lineage of microglia is different than the astrocytes and neurons. This is being actively investigated and remains to be established and explored.

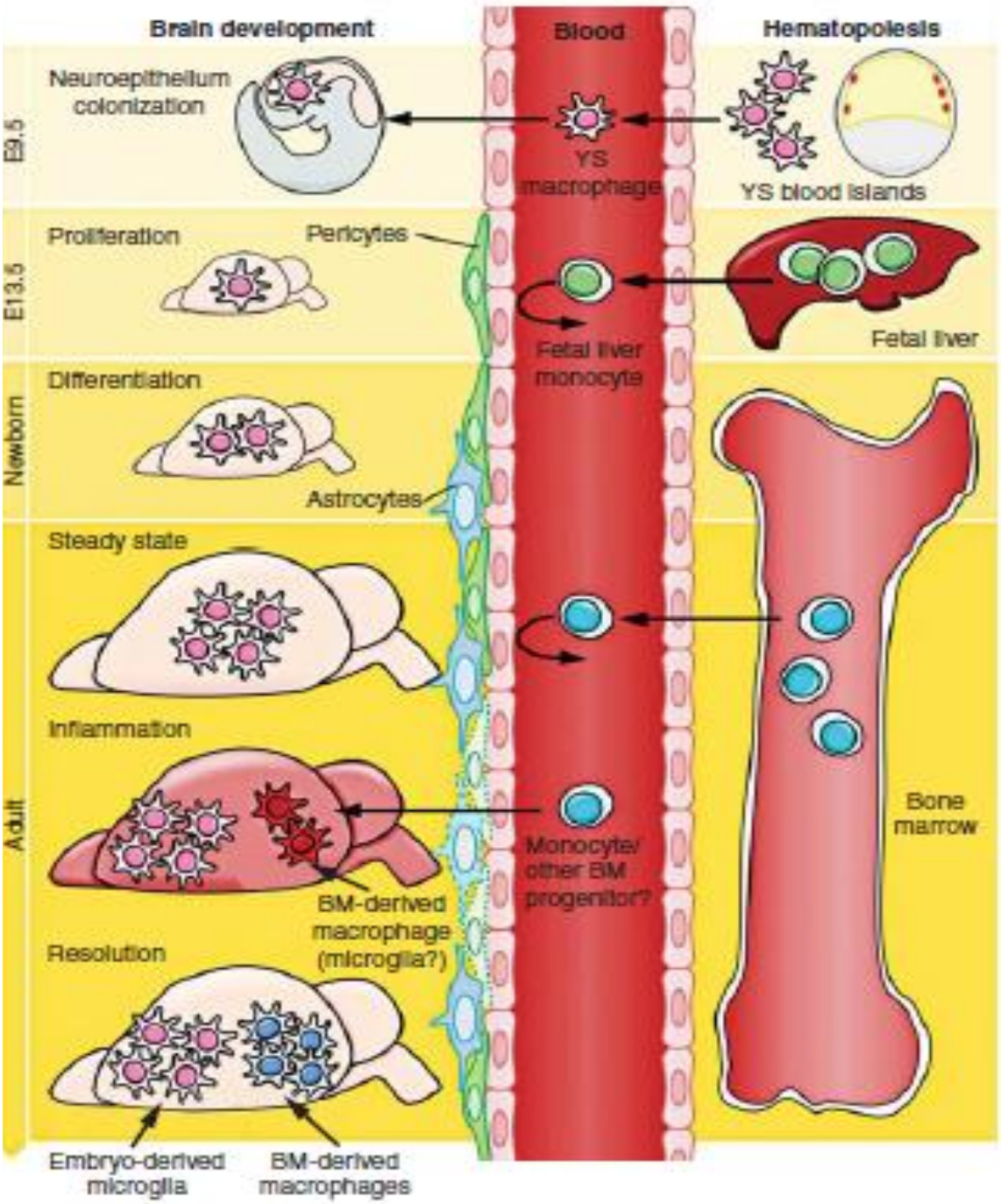

Brain development
Blood
Hematopoiesis
E9.5
Neuroepithelium colonization
YS macrophage
YS blood islands
E13.5
Proliferation
Pericytes
Fetal liver monocyte
Fetal liver
Newborn
Differentiation
Astrocytes
Adult
Steady state
Inflammation
Monocyte/ other BM progenitor?
Bone marrow
BM-derived macrophage (microglia?)
Resolution
Embryo-derived microglia
BM-derived macrophages

Figure2.7: Development of the microglia
(Ginhoux and Prinz, 2015)

2.4.2 Physiological states of microglia

During development, in the normal brain, and in the brain following injury, microglia display varied morphologies. The morphological differences in microglia are suggested to represent different functional states. Morphologically, microglia have three major transitional stages that can be distinguished as: amoeboid, ramified or resting and reactive or activated(Figure 2.8).

2.4.2.1Amoeboid microglia

Morphologically, they are round or irregular in shape and closely resemble macrophages(Figure 2.8). This shape allows the microglial free movement throughout the neural tissue, which allows it to fulfil its role as a scavenger cell. They are more prevalent during development, originate from the yolk sac and populate the developing brain early. Association of developmental neuronal cell death and microglia has been reported in most parts of the CNS (Pont-Lezica *et al.,* 2011)including the hippocampus, the cerebellum, the retina and the spinal cord (Marı´n-Teva *et al.,* 2004; Wakselman *et al.,* 2008 Caldero´ *et al.,* 2009; Rigato *et al.,* 2011). Their phagocytic as well as ability to induce apoptosis in unwanted neurons in developing brain makes them important participant in the process of CNS development. The phagocytic activity of microglia, involves the engulfing of various materials. Engulfed materials generally consist of cellular debris, lipids, and apoptotic cells in the non-inflamed state, and invading virus, bacteria, or other foreign materials in the inflamed state. Once the microglial cell is "full" it stops phagocytic activity and changes into a relatively non-reactive gitter cell.

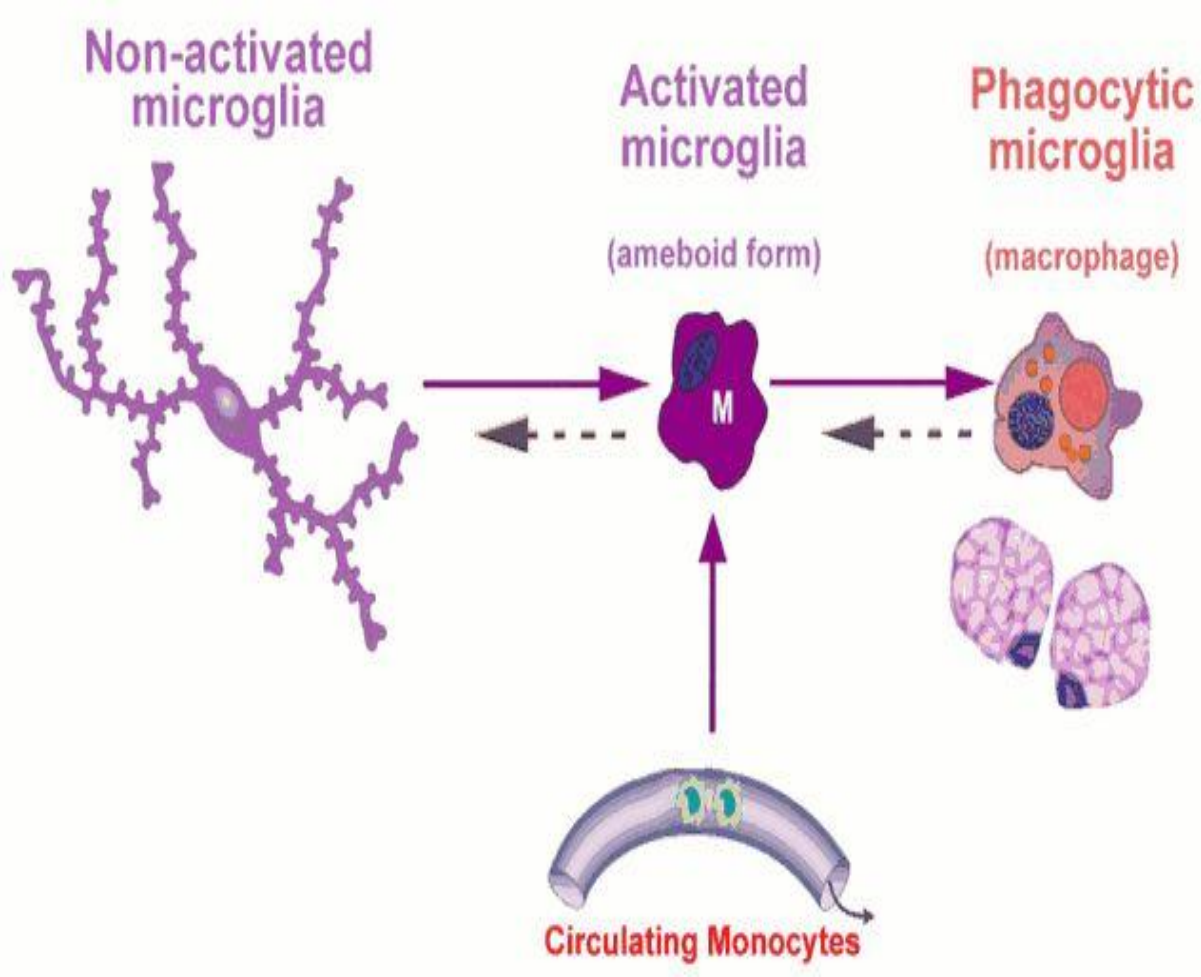


Figure2.8: Physiological states of microglia
(Ginhoux *et al.*, 2013)

Although amoeboid microglia are able to phagocytose debris, they do not fulfil the same antigen-presenting and inflammatory roles as activated microglia. They also interact with the synapses and modulate synaptic plasticity via pruning of excessive unwanted synapses and this is mediated by the complement pathway (Schafer *et al.,* 2012; Ginhoux *et al.,* 2013; Neiva *et al.,* 2014). Amoeboid microglia are generated from primitive myeloid/haematopoietic progenitor cells during the embryonic and perinatal stage and sustain up to the early postnatal stages in rats (Prinz and Mildner, 2011; Gomez *et al.*, 2013) and finally transform into ramified microglia (Kaur *et al.,* 1985).

2.4.2.2Ramified or resting microglia

This form of microglial cell is commonly found at specific locations throughout the entire central nervous system. This "resting" form of microglia is composed of long branching processes and a small cellular body(Ishan *et al.,* 2016). Unlike the amoeboid forms of microglia, the cell body of the ramified form remains in place while its branches are constantly moving and surveying the surrounding area (Ishan *et al.,* 2016). These processes extend into the brain microenvironment creating a matrix-like structure that helps to better perceive the CNS environment and detect very slight changes in physiological condition. Microglia are considered to be the critical effectors and regulators of changes in CNS homeostasis in health and disease as well as during CNS development (Prinz and Priller, 2014). Unlike activated or ameboid microglia, ramified microglia do not phagocytose cells and secrete fewer immunomolecules (including the MHC class I/II proteins).

Healthy neurons maintain microglia in an inactive state via secreted and membrane-bound signals, including CD200, CX3CL1 (fractalkine), neurotransmitters and neurotrophins (Neumann, 2001; Biber *et al.,* 2007; Pocock and Kettenmann, 2007). The expression of CD200 on neurons and endothelial cells in the CNS and the expression of its receptor, CD200R, predominantly on cells of myeloid origin, including macrophages and microglia, support a mechanism of neuronal/glia interactions to maintain microglia in a quiescent state (Neumann, 2001; Wright *et al.,* 2003; Lyons *et al.,* 2007). In this state they are able to search for and identify immune threats while maintaining homeostasis in the CNS. Although this is considered the resting state, microglia in this form are still extremely active in chemically surveying the environment (Christensen *et al.,* 2006). Ramified microglia can be transformed into the activated form at any time in response to injury or threat. (Verkhratsky and Butt, 2013).

2.4.2.3Reactive microglia

A multitude of signals that pose a potential threat to brain homeostasis are sensed by microglial receptors. Following any unfavourable stimuli ramified microglia get transformed into a reactive or activated state.In this state the processes of the resting microglia become retracted and the microglia takes on a round or irregular morphology (amoeboid)(Figure 2.8).Findings from *in vivo* and *in vitro* studies have reported that Pb exposure alters cellular functions in ways that might be expected to promote chronic microglial activation. Reactive microglia can either be non-phagocytic or phagocytic (Van Rossum and Hanisch, 2004; Hanisch and Kettenmann, 2007).

Non-phagocytic:

This state is actually part of a graded response as microglia move from their ramified form to their fully active phagocytic form. They generally appear as small amoeboid cells and

undergo rapid proliferation in order to increase their numbers (Aloisi, 2001; Christensen *et al.,* 2006).Specific factors released by stressed or damaged neurons have the potential to stimulate the production of pro-inflammatory cytokines by microglia. These include matrix metalloproteinase-3 (MMP-3), N-synuclein, neuromelanin, and adenosine triphosphate (ATP). Danger signals emitted by necrotic cells that can also stimulate similar responses include the heat shock proteins (HSP60, HSP70 and HSP90), DNA, proteases, uric acid, and the chromosomal protein high-mobility group B1 (HMGB1) (Kraft and Harry, 2011).

Microglia can also be activated by glutamate receptor agonists, pro-inflammatory cytokines, lipopolysaccharide, and changes in extracellular potassium (indicative of ruptured cells). In addition, microglia can detect ligands for CD40, CD91, and the intracellular NOD-like receptors (NLRs). These receptors initiate the signaling process by binding to pathogen associated molecular patterns (PAMPs). Ligation of PRRs leads to the activation of signal transduction pathways and regulation of diverse transcriptional and post-transcriptional molecules. These molecules include members of the nuclear factor kappa B (NF-OB), activator protein 1 (AP-1), and interferon regulator factor families, which modulate pro-inflammatory target genes encoding cytokines, chemokines, enzymes, and other molecules essential for pathogen elimination (Akira, 2006).

Depending upon the stimulus and progression of the diseased state, microglial activation acts in two ways; either help in efficacious restoration of the injured brain cells or generate a threatening environment that results in exaggerated brain damage(Ishan *et al.,* 2016). Once activated the cells undergo several morphological changes such as the thickening and retraction of branches, uptake of MHC class I/II proteins, expression of immunomolecules, secretion of cytotoxic factors, secretion of recruitment molecules, and secretion of pro-inflammatory signalling molecules (resulting in a pro-inflammation signal cascade) (Ishan *et al.,* 2016).

The ‘classical activation (popularly known as M1 phase)’ represents the initial innate immune response induced by Toll-like receptor (TLR) ligands and interferon-c (IFN-c) followed by the reactive release of a plethora of pro-inflammatory molecules like tumour necrosis factor-a (TNF-a), interleukin-6 (IL-6), interleukin-1b (IL-1b), interleukin-12 (IL-12), superoxide anions, nitric oxide synthase, redox molecules like nitrogen dioxide 2 (NOX2), nitrogen dioxide1 (NOX1), a member of Rho family of GTPases (RAC1), inducible nitric oxide synthase (iNOS), nitric oxide synthase 2 (NOS2) and excitotoxic molecules like group II metabotropic glutamate receptor (MGluR2), glutamate transporter-1 (GLT-1), purinergic P2X7 receptor (P2X7-R) (Benoit *et al.,* 2008).Superoxide, in particular, is implicated in microglial activation, cellular redox imbalance, and associated neurodegeneration. It is important to note that microglial production of superoxide exhibits significant species dependence as regards the quantity of superoxide produced in response to the same activating stimuli (Colton *et al.,* 1996).

The next alternate phase of microglial activation is ‘M2 or alternate activation’ phase, that dampen inflammation by switching over to the anti-inflammatory state by secreting molecules like interleukin-4 (IL-4), interleukin-3 (IL-3), interleukin-1 receptor antagonist (IL-1RA), scavenging receptors and extracellular matrix molecules (Luo and Chen, 2012). The cytoactive molecules thus released mitigate the generation of pro-inflammatory molecules. This accelerates the process of wound healing and damaged tissue repair (Martinez *et al.,* 2008). The third or subtype of M2 phase is ‘acquired deactivation’ associated with deactivation of glial inflammation and uptake of apoptotic cells or oxidized lipids via release of anti-inflammatory cytokines like transforming growth factor-b (TGF-b) and interleukin-10 (IL-10) (Gregory and Devitt 2004; Colton, 2009).

Phagocytic:

Activated phagocytic microglia are the maximally immune responsive form of microglia. These cells generally take on a large, amoeboid shape, although some variance has been observed. In addition to having the antigen presenting, cytotoxic and inflammatory mediating signalling of activated non-phagocytic microglia, they are also able to phagocytose foreign materials and display the resulting immunomolecules for T-cell activation (Gehrmann, 1995). Phagocytic microglia travel to the site of the injury, engulf the offending material, and secrete pro-inflammatory factors to promote more cells to proliferate and do the same. Activated phagocytic microglia also interact with astrocytes and neural cells to fight off the infection as quickly as possible with minimal damage to the healthy brain cells (Aloisi, 2001).

2.4.3 Role of microglia in the brain

2.4.3.1Developing brain

The development and maturation of the brain involves a continuous modification of synapses which involves synaptic pruning (pruning of inappropriate synapses) and strengthening of the established ones (Rakic and Zecevic, 2000). Paolicelli *et al.* (2011) suggested that activated microglia surround the regions undergoing developmental synapse turnover, and remove the unnecessary synapses in mice. The co-localization of neuronal postsynaptic protein within microglia cytoplasm suggested that microglia maintain the ability to engulf and remove dendritic spines during development. This specific phagocytosis may occur via signalling of the neuronal chemokine, fractalkine (CX3CL1), to its receptor on microglia (CX3CR1) (Hughes *et al.,* 2002) as microglia number was reduced and synaptic pruning delayed in the developing brain of mice deficient for CX3CR1 (Paolicelli *et al.*, 2011). Thus, any kind of deviation in microglial involvement leads to deficits in synaptic remodelling and maintenance, resulting in developmental disorders.

Microglia are also believed to be involved in regulation of neuronal proliferation and differentiation during development (Farinas *et al.,* 2002). Neurogenesis of embryonic cortical cells is enhanced in culture by microglia Jonakait *et al.,* (2000) and Aarum *et al.,* (2003) proposed that the exogenous addition of microglia to conditioned medium enhanced neurogenesis of embryonic cortical cells and the differentiation of basal forebrain progenitors into cholinergic neurons. Similarly, Antony *et al.,* (2011) suggested that microglia are an important component of embryonic neural precursor differentiation in the cortex. In the absence of microglia, proliferation of cortical precursors and astrogenesis is reduced, while an increase in microglia enhances the differentiation of cortical precursors into astrocytes.

Additionally, the release of soluble factors by microglia can serve to direct neural progenitor cell differentiation toward a neuronal phenotype, possibly via the expression of the innate pattern recognition receptors, toll-like-receptors (TLRs) (Rolls *et al.,* 2007). Microglia are observed within the myelin tracts during early development. Both inflammation and changes in white matter microglia have been associated with deficits in oligodendroglia progenitor cells or myelination as occurs with periventricular leukomalacia (Deng *et al.,* 2008; Harry *et al.,* 2006) or in receptor-dependent events. They are also involved in apoptosis (Miller and Kaplan, 2001) by producing neurotrophins (Nakajima *et al.,* 2001). Therefore, microglia undoubtedly play a supportive and directive role in both neurogenesis and gliogenesis in developing brain (Thored *et al.,* 2009).

Lead exposure impairs the hippocampal LTP and is suggested to be mediated by the alteration of glutamate-N-methyl-D-aspartate (NMDA) receptor system (Liu *et al.,* 2012. These findings fell into the conventional neuron-centered theory. Lead toxicity on neurons is commonly taken into consideration when investigating the Pb-induced LTP and learning and memory impairmentsbut its effect on glia cells have not been readily explored.Substantial

involvement of microglia in the Pb-induced hippocampal LTP impairment have also been reported. Although little is known about the effects of postnatal lead exposure on microglia activation in the hippocampus of developing Wistar rats.

Liu *et al.,* (2012) reported a significant increase in presence of activated microglia cells when compared to resting microglia in the hippocampus of weaning Sprague-Dawley rats (SD-rats) administered lead acetate for eight weeks. Post-natal exposure to lead at 30 ppm and 330ppm has also been reported to cause a significant decrease in the number of microglia cell in the dentate gyrus of young mice (Sobin *et al.,* 2013). Lead exposure was also reported by Liu *et al.*, (2015) to cause a significant increase in the number of microglial cells in the hippocampus of young mice treated with 15mg/kg of lead intraperitoneally for 3days. Lead have also been reported to elicit negative effects on the microglia cells. This may be as a result of disruption of this P2X7 which is critical for rapid proliferation of rapid proliferation of activated microglia cells (Sobin *et al.,* 2013).

2.4.3.2Adult brain

Ramified/resting microglia reside at strategic locations throughout the mammalian central nervous system (CNS). This microglia are continuously surveying the healthy brain for any disparaging condition at a speed of 1.47 μms^{-1} with their long thin processes (Nimmerjahn *et al.,* 2005). Recent *in vivo* studies have recorded the region specific speed of process motility to be between 0.2 and 6.5 μm/min (Tremblay *et al.,* 2010). During surveillance, microglial processes constantly establish a direct contact with neuronal synapses (Wake *et al.,* 2009).

During this period they release various neurotrophic growth factors to promote the neuronal survival and also to enhance neurogenesis (Ekdahl *et al.,* 2009).

In neurodegenerative diseases and following brain insults the resident microglia get stimulated and transform into activated or reactive state. In such situations microglia release numerous inflammatory molecules, growth factors, matrix proteins, chemokines and reactive free radicals which either initiate cellular repair or cytotoxic processes via interactions with other brain cells, e.g. astrocytes and neurons (Gomes-Leal, 2012). The role of microglia depends upon the type and intensity of the insult and associated microglia activation. Microglia are also important in both learning and synaptic remodelling (Parkhurst *et al.,* 2013). In adult they help in regulation of long-term potentiation (LTP) and tuning of synaptic strength, which is responsible for consistent long-term neural networks (Ben and Pascual, 2010; Kettenmann *et al.,* 2011). They maintain synaptic plasticity by releasing various soluble molecules responsible for regulating learning and memory and augmentation of N-methyl-D-aspartate (NMDA)-mediated LTP responses. Hayashi *et al.,* (2006) and Rogers *et al.,* (2011) predicted that the absence of microglia-mediated fractalkine receptor CX3CR1 signalling and secretion of glycine and L-serine by experimental intervention results in diminished learning and memory.

Moreover, microglia also mediate the modulation of GABAergic transmission and basal glutamatergic signalling via brain-derived neurotrophic factor (BDNF) and adenosine triphosphate (ATP) (Coull *et al.,* 2005; Pascual *et al.,* 2012). BDNF is required for tyrosine kinase B (TrkB) phosphorylation responsible for synaptic plasticity. Mice models depleted of microglia was seen to have impaired ability in multiple learning tasks and a reduction in motor learning-associated synaptic formation (Ishan *et al.,* 2016).

2.4.4Role of microglia in immune regulation

Following injury, inflammation, blood–brain barrier (BBB) damage (or metabolic disorder associated stimuli, microglia get activated (Ishan *et al.,* 2016). Microglia have also been activated exogenously by various inflammatory stimuli such as lipopolysaccharide, b-amyloid, interferon-c (Sharma *et al.,* 2015). Activated microglia release an array of immunocompetent molecules comprising of numerous chemokines like MIP-1a (Macrophage-Inflammatory Protein-1a), MIP-1b, MIP-2, MCP-1 (Monocyte chemoattractant protein-1), RANTES (regulated on activation, normal T cell expressed and secreted), IP-10 (IFN-c-inducible protein-10), and interleukins like (IL-3, IL-6, IL-10, IL-12, IL-18) tumour necrosis factor a (TNF-a), interferon gamma inducing factor (IGIF), inflammatory proteins, TGF-b (Ishan *et al.*, 2016). Collectively, these molecules not only control the inflammatory processes, but also regulate immune response of the brain and even contribute to neuropathogenesis in CNS inflammation.

Activated microglia also promote neuroprotection by releasing anti-inflammatory molecules and growth factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), basic fibroblast growth factor, following immunological stimuli (Hanisch and Kettenmann, 2007).

2.4.5 Resident microglia versus infiltrating, blood-borne monocyte

It is now generally acknowledged that all CNS disorders are characterized by microglial activation and that the progression and resolution of many diseases is contingent, in part, on the activity of microglia. In many of these clinical cases, such as stroke, head trauma, and advanced neurodegenerative disease, an associated disruption of the BBB is observed, allowing entry of cells from the bloodstream. In many of these conditions, it is possible that brain resident microglia are not contributing significantly to many of the observed

mononuclear cell effects. In addition to the secondary contribution of cells from the circulating blood, components of plasma such as fibrinogen can, in and of themselves, initiate a response in microglia (Adams *et al.,* 2007; Ransohoff and Perry, 2009; Ryu *et al.,* 2009).

Under normal conditions, following various pharmacological intervention/stimulants, or in the early stages of neurological disorders or neurodegenerative diseases, the job of "brain macrophage" falls to the resident microglia. However, with any compromise of the blood brain barrier, this task is accomplished by both the resident microglia and infiltrating blood borne monocytes. With the entry of blood-derived monocytes, the brain not only has multiple sources of phagocytic cells, or brain macrophages, but also gains a strong presence of antigen presenting cells (APCs) (Hickey and Kimura, 1988; Carson *et al.,* 1998).When peripheral monocytes enter the brain upon injury they assume the morphology and protein expression of microglia, thus, hindering the ability to discriminate between the two cell types of brain macrophages (Flugel *et al.,* 2001). In addition, the infiltration of peripheral monocytes, localized damage to the vasculature would allow access of activated pericytes. With a physical injury such as trauma or ischemia, or during autoimmune events, the barrier is disrupted and blood-borne immune cells gain access to the brain parenchyma, although this entry into the brain is limited and delayed as compared to peripheral tissues (Andersson *et al.,* 1992). As with an autoimmune disease, this entry can occur regardless of the presence or absence of tissue damage if the blood-brain barrier is compromised (Matsumoto and Fujiwara, 1987).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Ethical approval

Ethical approval was obtained from Ahmadu Bello University Ethics Committee on Animal Use and Care with approval number ABUCAUC/2018/047 (Appendix I).

3.1.2 Experimental animals

Nine (9) non pregnant and three (3) male Wistar rats weighing between 120g to 150g were obtained from the Animal House Centre, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria - Nigeria. The rats were housed in different rat cages, fed rodent chow and water *ad libitum*without administrationof lead for two weeks (14 days) to acclimatize them prior to experimental study.

3.1.3 Chemical procurement

3.1.3.1 Lead II acetate

Analytical grade of lead acetate of 99.9% purity with batch number (CAS 304-55-2) manufactured by Best of chemical limited, New York, USA was purchased from Hali brothers chemical and surgical suppliers store in Sokoto,Sokoto state, Nigeria.

3.1.3.2Tomato lectin

1mg Tomato lectin (Biotinylated *Lycopersicon Esculentum*) composed of 98% active conjugate and 99% lectin biotinylated with catalog number B-1175 and 1mg active conjugate of Horseradish peroxidase streptavidin with catalog number SA-5004 was purchased from Vector Laboratories Incorporation Burligame, CA, 94010 U.S.A.

3.1.4Other materials

The following materials were also used;

Digital weighing balance, Cages, Water bottles, Saw dust for bedding, Animal feed, Distilled water, Microtome, Light microscope, Glass slides, Cover slips, stains (H and E, Cresyl violet), Fixatives (Bouin's fluid), Specimen bottle, Dissecting kit, Beakers, Syringes, Anaesthesia (Ketamine), Centrifuge and Atomic Absorption Spectrophotometer.

3.2 Methods

3.2.1 Determination of oestrous cycle in Wistar rats

This was carried out adopting the method of Marcondes *et al.,* (2002) and Adebisi, (2008). During the acclimatization period, the nine non pregnant female Wistar rats were kept in the same cage to synchronize their oestrus cycle (Mcclintok, 1978). Two weeks after acclimatization, vaginal secretions were taken daily with a plastic pipette filled with normal saline by inserting the tip into the rat vagina, but not deeply. Vaginal fluid was aspirated and

placed on glass slides. A different glass slide was used for each of animals in separate cages. One drop of the secretion was collected with a clean tip from each rat. Unstained fluid was observed under a light microscope, without the use of the condenser lens, with 10x and 40x objective lenses. The proportion of the cell types recognized was used to determine the phase of the estrous cycle. A proestrus smear consisted of a predominance of nucleated epithelial cells, an estrous smear primarily contained anucleated cornified cells, a metestrus smear was made of the same proportion among leukocytes, cornified, and nucleated epithelial cells and a diestrus smear was dominated with leukocytes.

3.2.2 Mating of rats and confirmation of pregnancy

Once the female rats were in the proestrus phase, they were caged overnight with sexually matured male rats in ratio of 3 females to one male. A positive mating was confirmed by vaginal plug examination early in the morning of the day after mating by using a plastic pipette filled with normal saline. The vaginal was flushed with normal saline by inserting a plastic pipette into the rat vagina, but not deeply. After flushing the vaginal lumen with normal saline, the content was gently aspirated with a glass slide and was observed under the light microscope, without the use of the condenser lens, at 10x and 40 x objective lenses. The presence of sperm cells in the vaginal smear confirmed copulation (day 0 of pregnancy) (Marcondes *et al.,* 2002).

3.2.3 Experimental design

When the rats were confirmed pregnant, they were separated and kept in separate rat cages containing one dam each and her pups. While in separated rat cages, pregnant Wistar rats were randomly distributed into three experimental groups of three rats each, consisting of a

control group (1) and experimental groups (2 and 3)(Table 3.1). The dams in each of the three cages in each group gave birth to an average of five (5) pups and an average of fifteen (15) pups was gotten per group. The day of birth was taken as post-natal day 1 (PND 1). Based on the reported oral LD_{50} of lead acetate which is 600mg/kg body weight for Wistar rats (Sujatha *et al.,* 2011), all the pups of the experimental groups (2 and 3) were exposed to lead acetate via lactation from dams exposed to 10% and 15% of the LD_{50} of lead via oral gavage respectivelyfor three weeks (from post-natal day(PND) 1 – PND 21)(Table 3.1). The control group (1) was given distilled water throughout the experimental period. All experiments were done to minimize the number of animals used and was performed in accordance to the Committee for Ethics in Animal Experimentation of Ahmadu Bello University, Zaria, Nigeria.

Table 3.1: The grouping of experimental Wistar rats damsand treatment

GROUPS(n = 3)	Dams Treatment (dosage/kg body weight)	DURATION (PND)
1.	Control (Distilled water) 2ml/kg body weight	1-21
2.	Lead acetate (60mg/kg body weight)	1-21
3.	Lead acetate (90mg/kg body weight)	1-21

3.2.4 Drug administration

From PND 1 – PND 21, the pups of the dams in experimental groups (2 and 3) were exposed to lead acetate via lactation. Dams in Group 2 were administered 60mg/kg bwt (body weight) of lead acetate and Group 3 were administered 90mg/kg bwt of lead acetate via oral gavage(Table 3.1). The dams in the control group (1) were allowed to feed on fed rodent chow and administered distilled water at 2ml/kg bwt without lead acetate during the period of experiment(Table 3.1).All administration were done once daily using an oropharyngeal cannula and a 1ml calibrated syringe.

3.2.5 Body weight assessment

After administration, a total of ten (10) pups were selected at random from each group. Prior to the day of sacrifice, the ten pups per group were fasted overnight by separating them from their mothers.On the day of sacrifice (PND 22), the pups in each group were weighed using a

sensitive weighing balancemanufactured by 3Dresyns (EAN 2724293711341)with sensitivity of 0.01g in the Department of Human Anatomy, Ahmadu Bello University Zaria, Nigeria. The resultant weights were recorded.

3.2.6 Euthanasia

On post-natal day (PND 22), the ten (10) pups from each group were injected intraperitoneally with ketamine hydrochloride (75mg/kg body weight). Immediately the rats went to sleep, the 10 pups of eachgroup were decapitated and their brains were quickly harvested by making a mid-sagittal incision through the skull.

3.2.7Brain weight and Brain somatic index estimation

Immediately after excision of the brainsfrom the skullof the ten pups in each group, they were quickly weighed using a sensitive weighing balancemanufactured by 3Dresyns with sensitivity of 0.01g. After weighing the brain, the resultant weights were recorded.The brain somatic index was calculated from the formula below:

$$\frac{\text{Brain weight (g)}}{\text{Final body weight (g)}} \times 100$$ (Ramakrishna, 2014)

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3.2.8Quantification of lead deposit in the brain

After they were subjected to anaesthesia (ketamine at 75mg/kg bwt intraperitoneally), the brains of fourpups each from the control group (1) and experimental group (2 and 3) were quickly harvested by making a mid-sagittal incision through the skull. The harvested brains were then weighed using a sensitive weighing balance. After weighing, the harvested brains

were cut along their longitudinal fissure to separate them into halves. One half was weighed, minced and homogenized in cold phosphate buffer (pH 7.4) five times its weight in mls. The resultant homogenate was centrifuged at 4000rpm for 10minutes to remove cellular debris using a centrifuge machine (Made in England/Serial No: 846307) at the department of Human Anatomy, ABU, Zaria. The resultant supernatant was then stored in plain bottles for estimation of lead concentration. The Lead concentrations quantification of the supernatant was done by Atomic Absorption Spectrophotometry (Sharafi *et al.,* 2015) using Atomic Absorption Spectrophotometry-lamp standard (AAS-L) model AA 6800 Shimadzu Japan. This was carried out at the National Research Institute for Chemical Technology (NARICT), Basawa Road, Zaria, Nigeria.

3.2.8.1Atomic absorption spectrophotometry procedure

The procedure involved two major processes:

Digestion:

0.5 ml of the supernatant was digested by incubating it in 5 ml nitric acid and hydrochloric acid (HCL) in a fume hood cupboard. The samples were then heated at 100°C for 1 hour to obtain a clear solution. This starts with changes in colour, evaporation and emission of gas. The resultant solution was the allowed to cool for 15-20 minutes. Filter paper was then used to filter any insoluble compound if present. 60ml bottle was then used to pour digested sample. Distilled water was added to make 60ml. The sample was then transferred to the AAS-L for the lead quantification (Sharafi *et al.,* 2015).

Atomization:

The samples were now transferred to a beaker and taken up by the nebulizer. It was then mixed with acetylene gas. Once in the flame chamber the samples were atomized via the burning of the flame. Using a standard lamp for lead, lead components in the samples absorbed the light at a particular wavelength. Afterwards, the absorbed light was passed through a monochromator and identified by a detector. The concentration of lead was then determined through an absorbance-concentration gradient curve.

3.2.9Oxidative stress markers

Some of supernatant derived from the four pups wasalso stored in plain bottles for estimation of oxidative stress markers at the Department of Human Anatomy, Ahmadu Bello University Zaria, Nigeria.

3.2.9.1 *Superoxide Dismutase Activity*

This was determined according to the method of Fridovich, (1989).

Principle: This assay was based on the ability of superoxide dismutase to inhibit auto oxidation of adrenaline at pH 10.2.

Reagents:

0.5 M carbonate buffer: 14.3g of Na_2CO_3 and 4.2g of $NAHCO_3$ was dissolved in distilled water and made up to 1000ml in a volumetric flask. The buffer was adjusted to pH 10.2.

0.3M adrenaline: 0.01g of adrenaline was dissolved in 17ml of distilled water. The solution was prepared fresh.

Procedure:

0.1ml of the tissue homogenatewas diluted in 0.9ml of distilled water to make 1:10 dilution tissue homogenate. An aliquot mixture of 0.2ml of the diluted tissue homogenate was added to 2.5ml of 0.05M carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3mM adrenaline. The reference mixture contained 2.5ml of 0.05M carbonate buffer, 0.3ml of 0.3mM adrenaline and 0.2ml of distilled water. Absorbance was measured over 30s up to 150s at 450nm.

Calculations

Increase in absorbance per minute = (A5-A1)/2.5

$$\% \text{ inhibition} = 100 - \frac{\text{increase in absorbance for substrate}}{\text{increase in absorbance of blank}} \times 100$$ (Fridovich, 1989).

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to the tissue homogenate in 1 minute.

3.2.9.2 *Lipid peroxidation (Malondialdehyde)*

Lipid peroxidation was determined according to the method of Okhawa *et al.,* (1979).

Principle: Lipid peroxidation generates peroxide intermediates which upon cleavage release malondialdehyde, a product that reacts with thiobarbituric acid. Therefore it is determined based on thiobarbituric-acid reactive substance (TBARS)

Reagents: 15% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA).

Procedure: 1ml of 15% trichloroacetic acid was measured into a test tube. To that, 1ml of 0.67% thiobarbituric acid was added along with 50uL of the tissue homogenate. The mixture was incubated at 80^{o}c for 30minutes in a bath water and allowed to cool for some time followed by centrifugation at 3000rpm for 10mins. A clear supernatant was collected and the absorbance of it was determined at 535nm spectrophotometer.

TBARS concentrations were expressed in nmol/mg protein calculated as follows:

$$\text{Conc. Nmols/mg protein} = \frac{\text{absorbance of sample}}{1.56 \times 10^{-5} \times \text{protein conc. (mg)}}$$ (Rajagopalan *et al.*,2004)

3.2.9.3 *Assessment of reduced glutathione*

This was determined according to the method of Ellman, (1959) as described by Rajagopalan *et al.*, (2004).

Principle: The principle of assay is based on the reaction of 5, 5'-dithiobis nitrobenzoic acid (DNTB) and reduced glutathione (GSH).

Reagents:

<u>0.2M phosphate buffer:</u> 8.40g of NaH_2Po_4 were dissolved in distilled water and made up to 1000ml make in a volumetric flask. The buffer was adjusted to pH8.0.

<u>10% Trichloroacetic acid (TCA):</u> 10g of TCA was dissolved in distilled water and made up to 100ml in the volumetric flask.

Ellman's reagent: 19.8g of 5, 5'dithiobis nitrobenzoic acid in 100ml of 0.1% sodium.

Procedure:

To 150uL of tissue homogenate in phosphate saline (pH 7.4), 1.5ml of 10% TCA was added and centrifuged at 1500g for 5 minutes. 1ml of the supernatant was treated with 0.5ml of Ellman's reagent and 3ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412nm and quantity of GSH was obtained from the graph of the GSH standard curve.

3.3 Histology and Histochemistry

The other half of the separated brain was weighed and the hippocampus was isolated according to the method of Olatomide andMusa, (unpublished 2018) and immediately fixed in Bouin's fluid for fixation. It was then processed routinely, sectioned at 10um thickness, and stained using H &E and Cresyl fast violet method (Freida, 2007).

3.3.0.1Fixation

The isolated hippocampus from the rats were fixed rapidly in freshly prepared Bouin's fluid solution to forestall bacteria decomposition, tissue decay and maintain the structure of the cell and its subcellular components (cellular organelles).

3.3.0.2Tissue processing

The fixed hippocampus was taken through the routine process of tissue processing (dehydration, clearing and infiltration) to remove the water from the tissues and replace with a medium that solidifies to allow thin sections to be cut (Winsor, 1994).

Dehydration – the fixed hippocampus samples were passed through increasing grades of alcohol (from 70% to 90% and to absolute or 100%) to remove the water present – either bound or free in the tissue.

Clearing – after dehydration, the samples of the hippocampus were placed in different changes of Xylene to clear the alcohol content in the tissue thereby making the tissue transparent and clear (Winsor, 1994).

Infiltration – after clearing, the hippocampus samples were transferred and immersed into a paraffin molten wax which replaces the Xylene and infiltrates the tissue sample.

3.3.0.3Embedding

After the hippocampus samples have been processed successfully, they were transferred into a flat metal mould with the molten paraffin wax. The samples in the metal moulds with the molten paraffin wax was allowed to cool and solidify for 24-48 hrs prior to formation of tissue blocks (Winsor, 1994).

3.3.0.4Sectioning

The tissue blocks formed after external embedding were cut and trimmed into thin sections at 10ῳm using a rotatory microtome. The sections were introduced into a water bath (at 50ºc to spread the ribbons) and the placed on microscopic slides and finally dried briefly in the oven (Winsor, 1994).

3.3.0.5Staining of slides

The tissue sections on each microscopic slide were finally stained for histological study (using the procedures of the Haematoxylin and Eosin staining techniques), histochemical study (using the procedures of Cresyl fast violet technique).

3.3.0.6 Haematoxylin and eosin staining techniques

The architecture of the hippocampus was determined by using the procedure of Ehrlich's (Ehrlich, 1886). The hippocampus sections were dewaxed inthree changes of xylene for 5 minutes each.Afterwards they were then hydrated by treating them with absolute alcohol (100%), 90% alcohol and 70% alcohol for three minutes each. They were now stained in haematoxylin for 10 minutes, washed well in running water for 2 – 3minutes and differentiated in 1% acid alcohol (1% Hcl in 70% alcohol) for 1 minute (this makes the blue staining turn red due to the action of the acid). The sections were now blued (to regain the blue colour) by washing in alkaline running tap water (Scott's tap water) for 10-20 minutes. Afterwards they were rinsed in water, stained in 1% of aqueous eosin for 3 minutes and rinsed again in water (to wash excess stain). The sections were now dehydrated in alcohol (70%, 90%, and absolute for 2 minutes each). They were then cleared in xylene for 2 minutes and finally mounted in synthetic DPX. The nucleus of the pyramidal cells stained blue/purple while the cytoplasm stained pink.

3.3.0.7 Cresyl fast violet (Nissl) stain

The hippocampus sections were dewaxed in three changes of xylene for 5 minutes each and were now rinsed in water. The sections were then covered with filtered cresyl fast violet stain for 10-20 minutes. Afterwards they were rinsed in distilled water, differentiated in 0.25% acetic alcohol until most of the stain has been removed. Afterwards they were briefly passed through absolute alcohol into xylene and checked microscopically. When proper staining was

confirmed, they were then rinsed well in xylene and mounted in DPX mountant (Bancroft and Gamble, 2008). Nissl substance within the perikaryon of the pyramidal cells stained violet.

3.3.1 Tomato lectin staining technique

The tomato lectin staining procedure was carried out according to the method of (Villacampa *et al.,* 2013)

3.3.1.1Fixation

Perfusion fixation:

The heart of anesthetized Wistar rat pups were accessed by making an incision through the abdomen, then through their diaphragm and the inferior vena cava (IVC) was then cut. The pups were first intracardially perfused with normal saline for 5 minutes to drain out blood. They were now perfused with paraformaldehyde fixative at 4 °C for 10 minutes. After perfusion fixation, the hippocampus was isolated out as quickly as possible and immersed in glass vials containing paraformaldehyde fixative(4 °C) to complete a total time of 4 hours of fixation. After the 4 hours of fixation, the samples of the hippocampus were washed in cold phosphate washing buffer (PWB) 4°C for 10 minutes.

3.3.1.2Tissue processing, embedding and sectioning

After fixation, the hippocampus samples were taken through the routine process of tissue processing (dehydration, clearing and infiltration) to remove the water from the tissues and replaced with a medium that solidifies to allow thin sections to be cut. After the hippocampus samples have been processed successfully, they were embedded and sectioned as stated above. They were then collected using positive charged slides and stored at room temperature (RT) until their use for the tomato lectin (TL) staining procedure (Villacampa *et al.,* 2013).

3.3.1.3 Staining

The hippocampus sections were dewaxed in three changes of xylene for 10 minutes each.They were now hydrated by immersing them in decreasing grades of ethanol (from 100%, 95 %, 70 %,50 %, and 30 %) for 1 minute each. Afterwards they were immersed in PBS for 2 min.The sections on the positive charged slide were then washed with Tris-buffered saline solution (TBS) at room temperature (RT) for 30 minutes. The sections were now incubatedin endogenous peroxidase blocking solution (EPBS) at RT for 10 minutes.Subsequently they were washedfor 30 minutes with Tris-buffered saline solution with Triton X-100 (TBST) at RT, incubatedwith the Tomato lectin (TL) diluted in TBST (1:150) overnight at 4 °C and afterwards 1hour at RT. The sections were now washed in TBST at RT, incubatedwith horseradish peroxidase streptavidin diluted in TBST (1:500) for 1 hour at RT and washed the sections 30 minutes in TBS at RT and washed for 30 minutes in Tris-buffer solution (TB) at RT. The sections were incubated in the developing solution (DS) for exactly 3 minutes (to visualizethe peroxidase reaction) and submerged in distilled water for 3 minutes.Afterwards they were counterstained with toluidine blue for 1 minute, washed with distilled water for 1 minute to remove the excess of colorant and dehydratedno more than 30 seconds by submerging the slides in graded ethanol: 50, 70, 90, and in three changesof 100 % ethanol. They were then differentiated with 1-butanol for 1 minute and submerged in two changes of xylene for 2 minutes each. Finally the slides were coverslipped using DPX as permanent mounting medium.The resting microglia stained brown, while the activated microglia stained dark brown.

3.3.2 Photomicrography

After staining, microscopic slides were viewed under the Motic SFC-100FL Monocular Transmitted Light Microscopemanufactured by Leica Camera AG at x250 and x1000 (oil

immersion) magnifications respectivelyand photomicrographs were taken using MD900 Amscope® digital camera.

3.4 Stereology

3.4.1Volume estimation

The absolute volume of the hippocampus was estimated using the Cavalieri estimator of volume according to the method of West, (1999). To achieve this, the hippocampus of two Wistar rat pups per group wereisolated, processed and sectioned serially using a microtome, as shown by Gundersen and Jensen, (1987) to provide 10 sections (10µmthick) after pilot study on how many slices could be derived. After sectioning the hippocampus at 10µm, a total of 30 sections was derived. Tissue sections of the hippocampus were then selected at every third using the systematic uniform random sampling method. The sections derived

were stained using tomato lectin. A transparent counting grid was placed randomly over the cut surface of every hippocampus slice at a magnification of (X10). The number of points hitting the hippocampus was counted (Appendix II). The volume was estimated using the Cavalieri'sprinciple(Gundersen *et al.,* 1988) as follows:

$$V := T \cdot (a/p) \cdot \Sigma p$$

where ''**:=**'' indicates that the result is the estimated value rather than the true value, "V" is the total volume of hippocampus, "T" = 0.03mm is the average slice thickness, "a/p" ¼ is the area associated with each point inthe counting grid (4mm²), and "Σp"is the total number ofpoints hippocampus.

Coefficient of error (CE) was calculated as follows:

$$CE = \frac{\sqrt{\text{Total variance}}}{\Sigma p}$$ (Gundersen *et al.,* 1988)

This was carried out at the Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria.

3.4.2Number of microglia cells

An unbiased estimate of the number of activated microglia in the hippocampus of Wistar rat pups was obtained using the physical fractionator method described by Pakkenberg and Gundersen, (1988) as reported by Kiymet *et al.,* (2018). To achieve this, the hippocampus oftwo Wistar rat pups per group was isolated, processed and sectioned at 10ɥmafter a pilot study on how many slices could be derived. After sectioning the hippocampus at 10ɥm, a total of 30 sections was derived.Tissue sections of the hippocampus were then selected at every third using the systematic uniform random sampling method. A total of ten (10) sections were derived from each hippocampus. The sections derived were then stained using

tomato lectin to demonstrate the microglia cells. To count the number of activated microglia cells, a transparent counting frame with acceptance and rejection region was used on two consecutive sections (Appendix III). One of the section planes was taken as the "look up" section and the other as the "reference" section. Activated microglia cells were selected in the reference section and counting was done in the look up section. If an activated microglia cell appears in the reference section but not the lookup section, then the particle is counted. The total number of the activated microglia cells was then computed as follows:

$N = Nv \bullet V(ref)$

Where $Nv = \frac{\Sigma Q^-}{2 \bullet 5 \bullet V(dis)}$, $V(dis) = T \bullet$ area of frame ΣQ^- = total number of cells counted (Gundersen *et al.*, 1988)

Reference volume V (ref) was derived from the estimated volume calculated.

Coefficient of error (CE) was calculated as follows:

$CE = \frac{SEM}{MEAN\sqrt{n}}$ Where SEM (standard error of mean) = $\frac{\text{Standard deviation (SD)}}{}$

n= 10 (number of hippocampus sections)(Gundersen *et al.*, 1988)

This was carried out at the Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria.

3.5Data Analysis

Data obtained from the study were expressed as Mean±SEM. One way analysis of variance (ANOVA) was used to compare the mean differences between and within the groups followed by LSD post-hoc test. P-value less than 0.05 was considered statistically significant. Data was analyzed using statistical package for social sciences (SPSS v. 20).

CHAPTER FOUR

4.0 RESULTS

4.1 Body Weight, Brain Weight and Brain Somatic Index

4.1.1 Body weight of Wistar rat pups from dams exposed lead acetate

There was a significant ($p<0.05$) decrease in body weight of Wistar rat pups in group 2 (16.53±0.28g) and group 3 (15.71±0.35g) exposed to lead acetate via lactation from dams administered 60mg/kg and 90mg/kg bwt of lead acetate respectively from PND 1-21 when

compared with the control (21.16±0.56g) (Table 4.1). The decrease in body weight of the pups in the group 3 (15.71±0.35g) administered 90mg/kg bwt of lead acetate when compared to group 2 (16.53±0.28g)treated with 60mg/kg bwt lead acetate via lactation from PND 1-21 was not significant ($p>0.05$) (Table 4.1).

4.1.2 Brain weight of Wistar rat pups from dams exposed to lead acetate

Brain weight of Wistar rats pups in group 2 (1.09±0.03g) and group 3 (1.13±0.02g) exposed to lead acetate via lactation throughdamsfrom PND 1-21 were insignificantly ($p>0.05$)lowerthan that of the control (1.15±0.04g) (Table 4.1).

4.1.3 Brain somatic index of Wistar rat pups from dams exposed to lead acetate

The brain somatic index significantly ($p<0.05$) increased in Wistar rats pups of group 2 (0.066±0.002) and group 3 (0.072±0.001) exposed to lead acetate via lactation from dams administered 60mg/kg and 90mg/kg bwt of lead acetate respectively from PND 1-21 when compared with the control (0.054±0.002)(Table 4.1). Between the groups, the brain somatic index also significantly ($p<0.05$) increased in pups of group 3 (0.072±0.001) when compared to group 2 (0.066±0.002)from PND 1-21 (Table 4.1).

Table 4.1: Mean body weight, brain weight and brain somatic indexof Wistar rat pups exposed to Lead acetate via lactation.

Values in Mean±SEM. One way ANOVA test followed by LSD post hoc test. Cells carrying different superscripts ($^{a, b, c}$) on each row are significantly different ($p<0.05$). PND - post natal day. n = 10. Pb – Lead. Dist. Water – Distilled water.

Parameters	(2 ml/Kg Dist. water)	(60mg/kg Pb) PND 1-21	(90mg/kg Pb) PND 1-21	*F*	*P*
Body Weight (g)	21.16±0.56[b]	16.53±0.28[a]	15.71±0.35[a]	50.29	<0.001
Brain Weight (g)	1.15±0.04	1.09±0.03	1.13±0.02	0.94	0.403
Brain Somatic index	0.054±0.002[a]	0.066±0.002[b]	0.072±0.001[c]	32.40	<0.001

4.2 Lead Concentration in brain tissues of pups from dams exposed to lead acetate

Lead accumulation in brain tissuesin Wistar rat pups of group 2 (0.04±0.01ppm) and group 3 (0.11±0.03ppm) from PND 1-21 were significantly ($p<0.05$) higher than the values obtainedin the control (-0.05±0.02 ppm)(Table 4.2). The lead concentration in the brains of the pups from group 3 (0.11±0.03 ppm)were also significantly ($p<0.05$) higher than the values obtained for pups from group 2 (0.04±0.01ppm) during the experiment (Table 4.2).

Parameters	(2 ml/Kg Dist. water)	(60mg/kg Pb) PND 1-21	(90mg/kg Pb) PND 1-21	*F*	*p*

Table 4.2: Lead concentration in Wistar rat pups exposed to lead acetate via lactation.

Lead Concentration (ppm)	-0.05±0.02[a]	0.04±0.01[b]	0.11±0.03[c]	16.515	0.001

Values in Mean±SEM. One way ANOVA test followed by LSD post hoc test. Cells carrying different superscripts ([a, b, c]) on each row are significantly different ($p<0.05$). PND - post natal day. n = 4 Pb – Lead. Dist. Water – Distilled water.

4.3 Oxidative Stress Biomarkers

4.3.1 Mean superoxide dismutase levels in Wistar rat pups from dams exposed to lead acetate postnatally

There was a significant ($p<0.05$) decrease in superoxide dismutase (SOD) levels in Wistar rats pups of group 2 (17.90±0.25 U/ml) and group 3 (16.50±0.46 U/ml) exposed to lead acetate via lactation from dams administered 60mg/kg and 90mg/kg bwt of lead acetate respectively from PND 1-21 in comparisonto the control(20.60±0.56 U/ml). The level of SOD in the pups of group 3 was insignificantly ($p>0.05$) higher than group 2 during the experimental period (Figure 4.1).

4.3.2 Mean reduced glutathione levels in Wistar rat pups from dams exposed to lead acetate postnatally

There was a significant ($p<0.05$) decrease in reduced glutathione (GSH) level in Wistar rats pups of group 3 (11.40±0.99 ug/ml) exposed to lead acetate via lactation from dams administered 90mg/kg bwt of lead from PND 1-21 when compared with the control(16.50±1.48 ug/ml). (Figure 4.2). The level of GSH in pups of group 2 (14.58±0.30 ug/ml) exposed to lead acetate via lactation from dams administered 60mg/kg bwt of lead acetate from PND 1-21 was insignificantly ($p>0.05$) lower than control (Figure 4.2).

4.3.3 Mean malondialdehyde levels of Wistar rat pups from lead acetate exposed dams

Malondialdehyde (MDA) levels increased significantly ($p<0.05$) in Wistar rats pups of group 3 (30.23±1.96 nmol/mg) exposed to lead acetate via lactation from dams administered 90mg/kg bwt of lead acetate from PND 1-21 when compared with the control (23.07±0.63 nmol/mg)and group 2 (25.20±0.80 nmol/mg) Wistar rats pups exposed to lead acetate via lactation from dams administered 60mg/kg bwt.

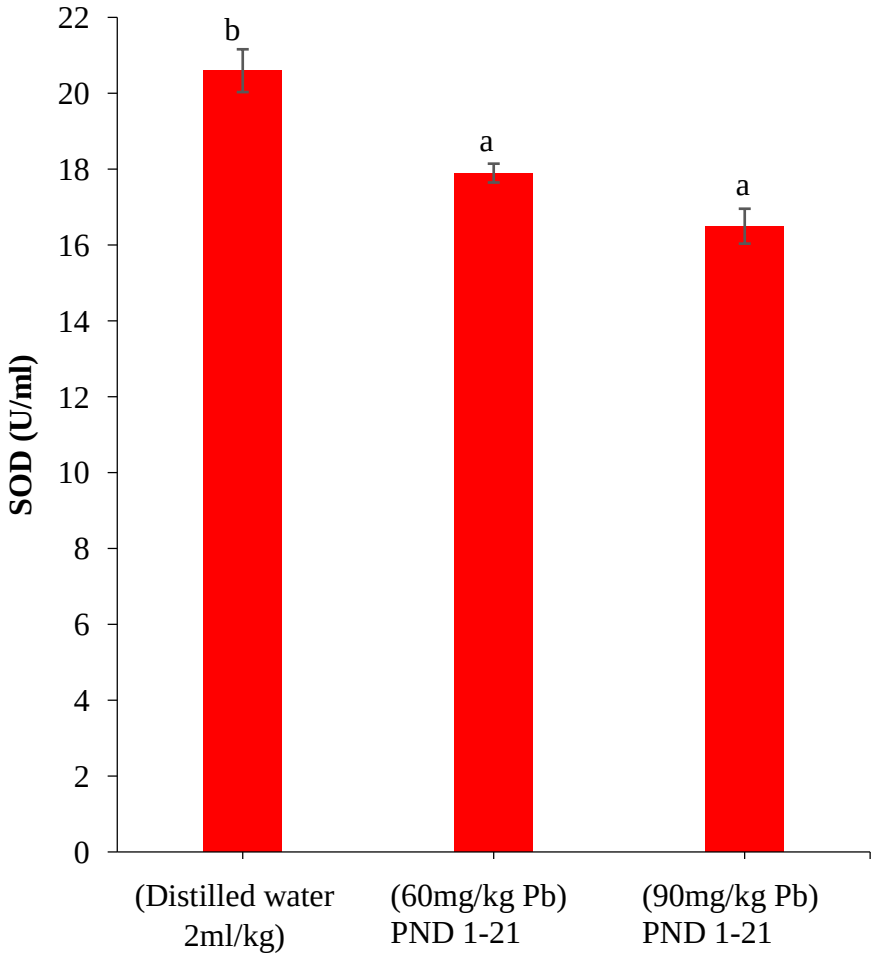


Figure 4.1: Mean levels of superoxide dismutase (SOD) of lead acetate treated Wistar rat pups. Bars carrying different superscripts ($^{a, b}$) are significantly different ($p<0.001$).PND - post natal day. n = 4. Pb - Lead

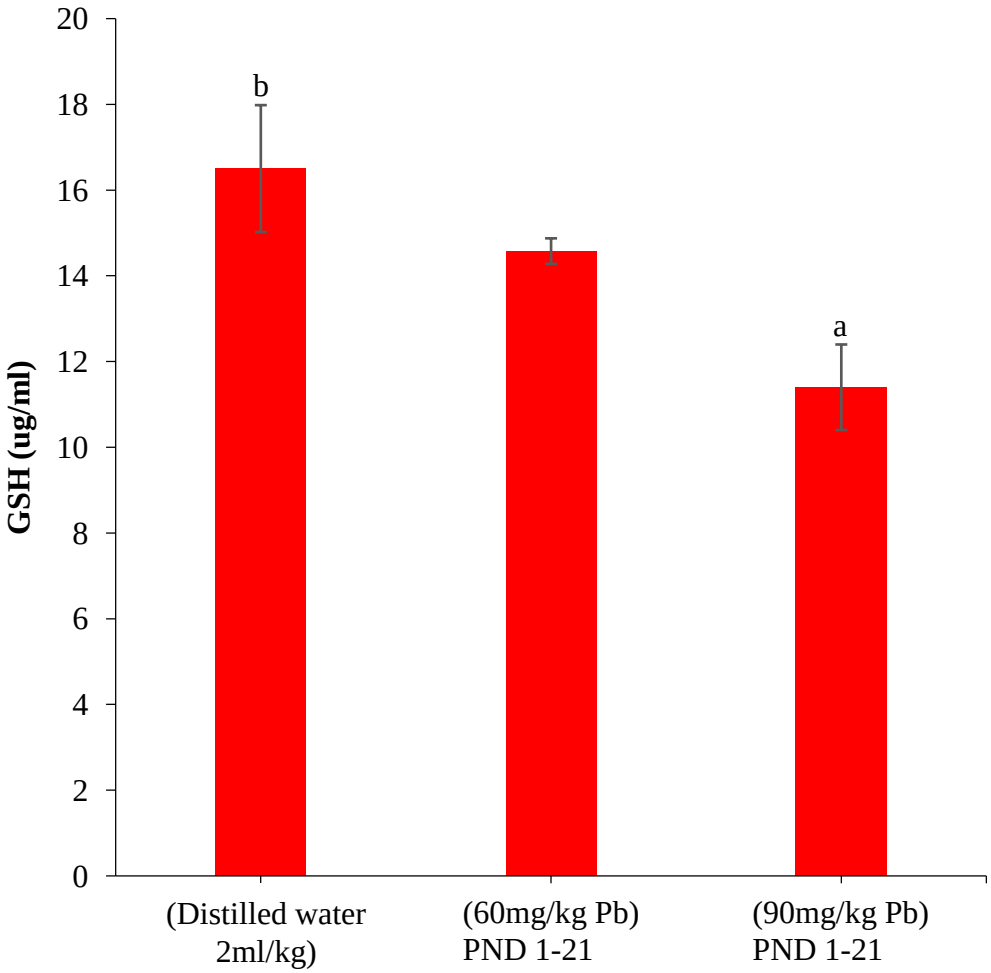


Figure 4.2:Mean levels of reduced glutathione(GSH) of lead acetate treated Wistar rat pups. Bars carrying different superscripts ($^{a, b}$) are significantly different (p=0.02).PND - post natal day. n = 4.Pb - Lead

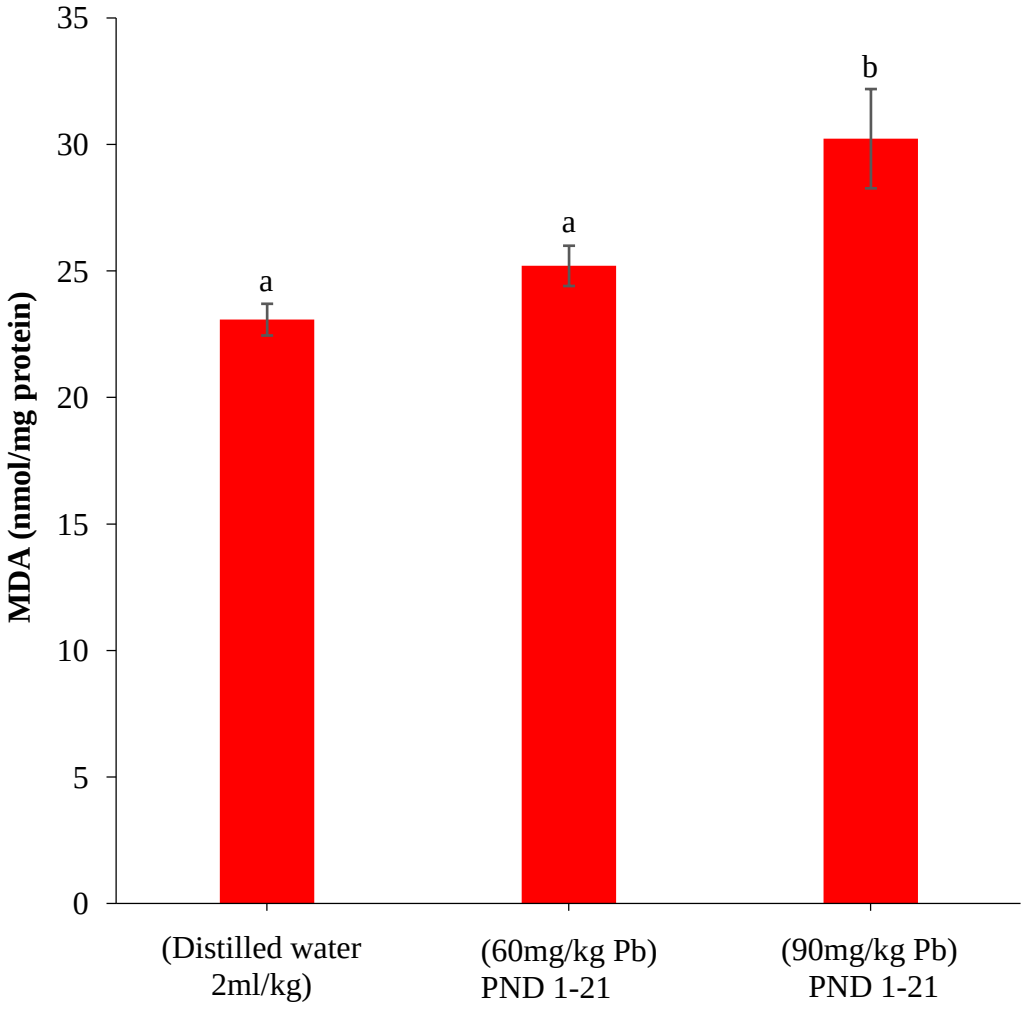


Figure 4.3: Mean levels of Malondialdehyde (MDA) of lead acetate treated Wistar rat pups. Bars carrying different superscripts ($^{a, b}$) are significantly different (p=0.01).PND - post natal day. n = 4. Pb - Lead

4.4Histological Studies

4.4.1 Haematoxylin and eosin (H and E) stain

The photomicrograph from the H and E stain of the CA3 region of the hippocampus of Wistar rat pups in group 1 (control group) administered distilled water revealed normal cytoarchitecture and characteristic appearance of the pyramidal cell with well-preserved cytoplasm, prominent nuclei and nucleoli and axon radiating from the cell body. It also showed moderately dispersed neuroglia cells (Plate I). The CA3 region of the hippocampus of Wistar rat pups in group 2 exposed to lead acetate via lactation from dams administered 60mg/kg of lead acetate from PND 1-21 stained with H and E stainrevealed distortion in cytoarchitecture of the hippocampus with presence of cell loss, inflammatory cells, cytoplasmic vacuolation and degeneration of pyramidal cells (Plate II). The photomicrograph of the CA3 region of the hippocampus in group 3 Wistar rat pups exposed to lead acetate via lactation from dams administered 90mg/kg of lead acetate from PND 1-21, showed distortion in cytoarchitecture with presence of more necrotic pyramidal cell, more inflammatory cells, pyknotic nucleus and clumping of cells(PlateIII).

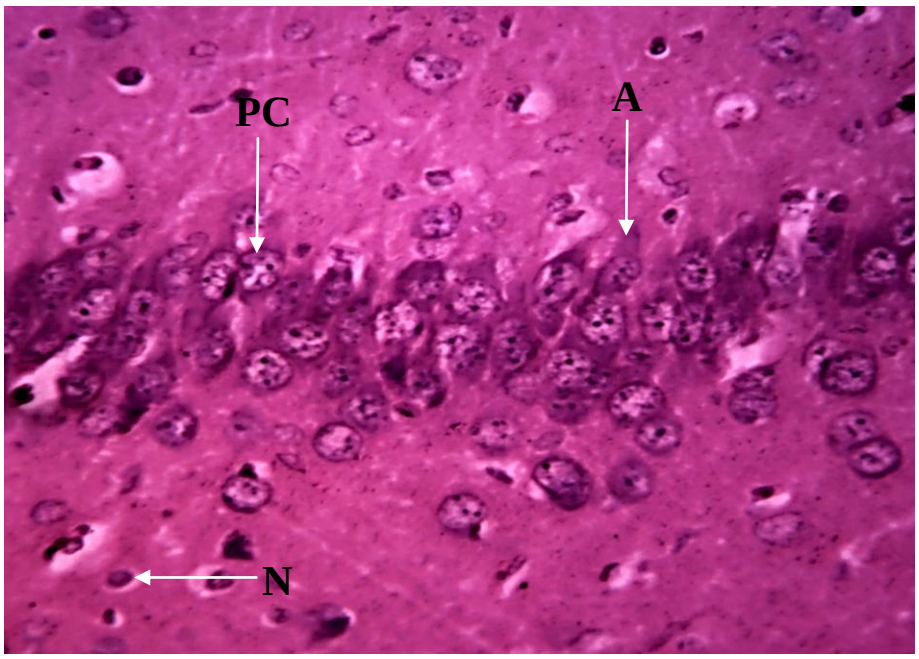


Plate I: Photomicrograph of the CA3 region of the hippocampus of Wistar rat pups in Group 1 (control group) administered distilled waterwith normal cytoarchitecture (H and E MagX250).

PC - Pyramidalcell
A - Axon
N - Nuclei of glia cell

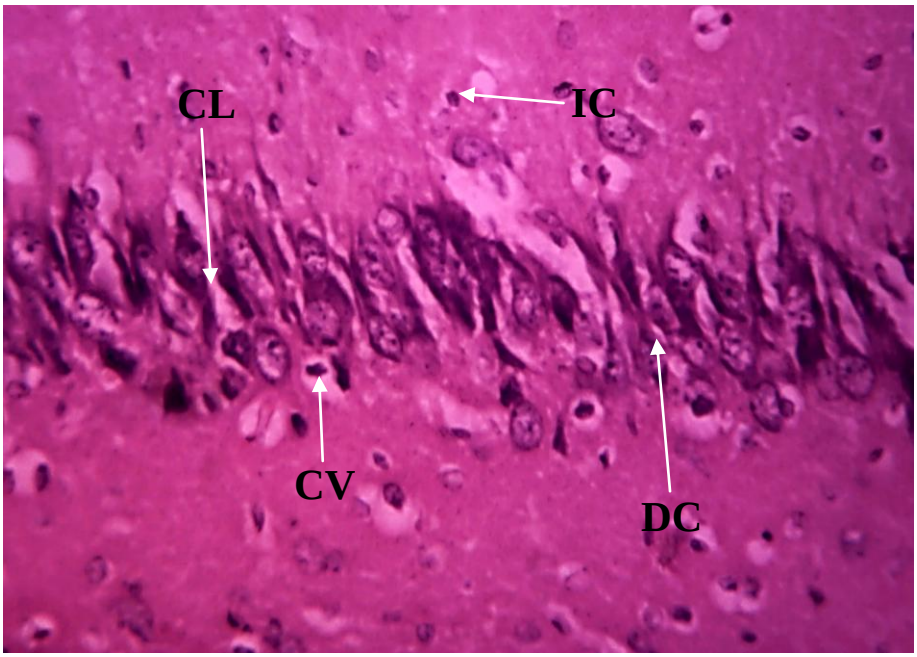


Plate II: Photomicrograph of the CA3 region of the hippocampus of Wistar rat pups in Group 2 exposed to lead acetate via lactation from dams administered 60mg/kg of lead acetate from PND 1-21. (H and E MagX250).

CL - Cell loss
CV - Cytoplasmic vacuolation
DC – Degenerating pyramidal cell
IC – Inflammatory cell

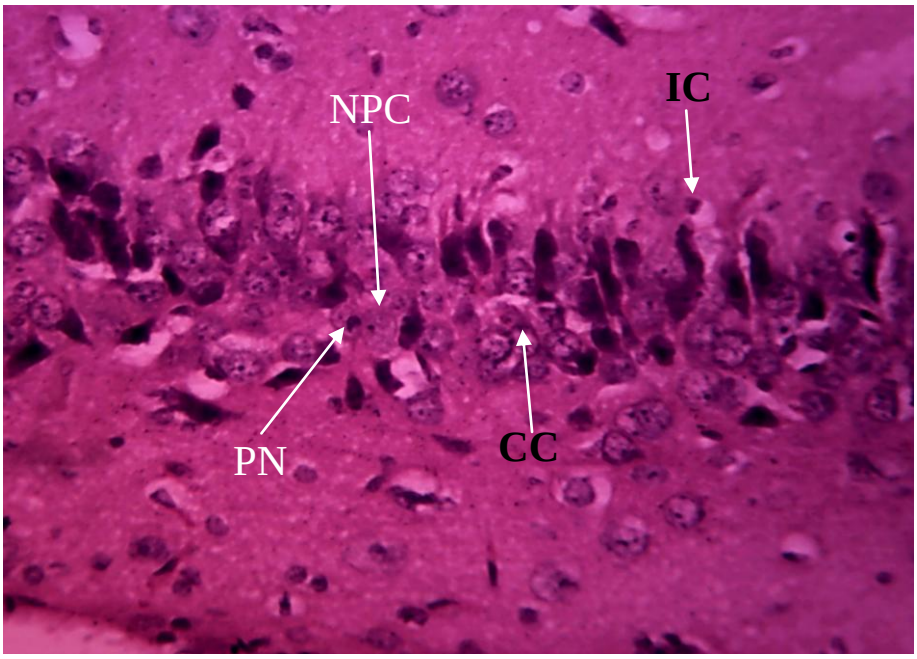


Plate III: Photomicrograph of the CA3 region of the hippocampus of Wistar rat pups in Group 3 exposed to lead acetate via lactation from dams administered 90mg/kg of lead acetate from PND 1-21(H and E MagX250).

CC - Clumping of cells
NPC – Necrotic pyramidal cells
IC - Inflammatory cells
PN - Pyknotic nucleus

4.4.2 Cresyl violet staining

The CA3 region of the hippocampus of Wistar rat pups in Group 1 (control group) administered distilled water revealed intense staining of the basophilic ribosomes in the perikaryon of the pyramidal cells (Nissl substance) after cresyl violet staining (Plate IV). The photomicrograph of the CA3 region of the hippocampus of Wistar rat pups in Group 2 exposed to lead acetate via lactation from dams administered 60mg/kg of lead acetate from PND 1-21 showed weak staining of Nissl substance when compared with the control but more intense staining when compared with group 3 as seen in (Plate V). The CA3 region of the hippocampus of Wistar rat pups in Group 3 exposed to lead acetate via lactation from dams administered 90mg/kg of lead acetate from PND 1-21 revealed very weak staining for Nissl substance when compared with the control (Plate VI).

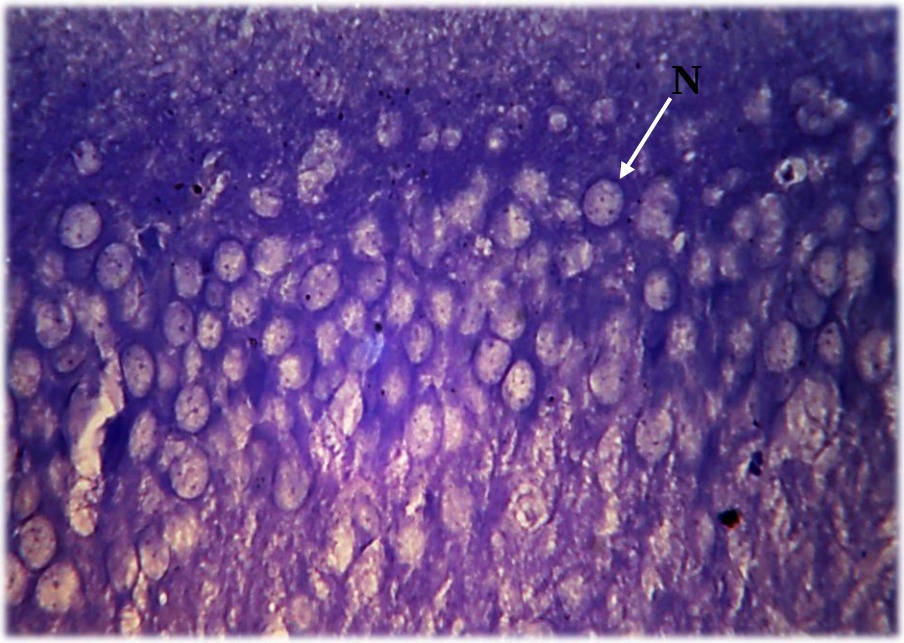


Plate IV: Photomicrograph of the CA3 region of the hippocampus of Wistar rat pups in Group 1 (control group) administered distilled water with intense staining for Nissl substance. (Cresyl violet stain MagX250).

N – Nissl substance

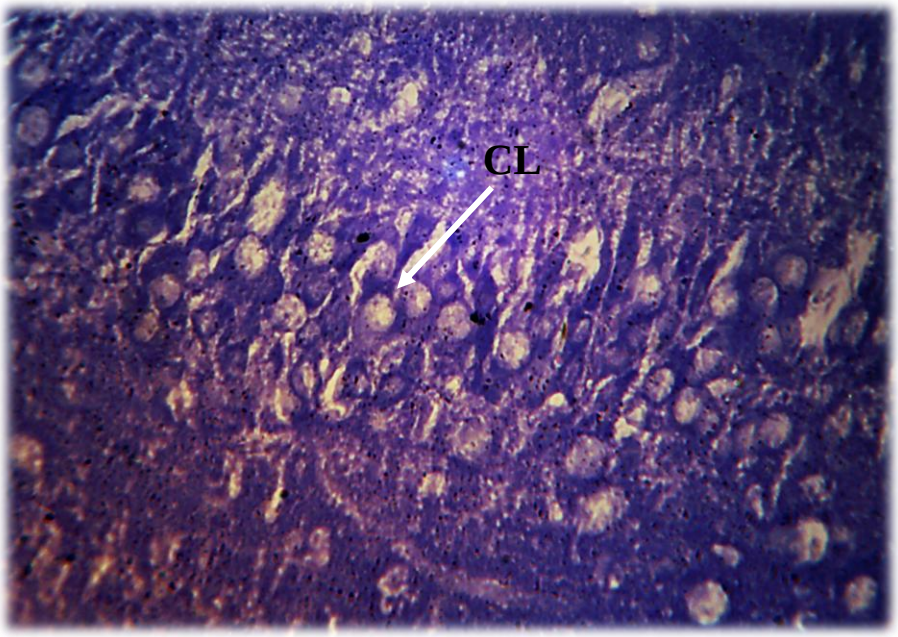


Plate V: Photomicrograph of the CA3 region of the hippocampus of Wistar rat pups in Group 2 exposed to lead acetate via lactation from dams administered 60mg/kg of lead acetate from PND 1-21 with weak staining andchromatolysis. (Cresyl violet stain MagX250).

CL- Chromatolysis

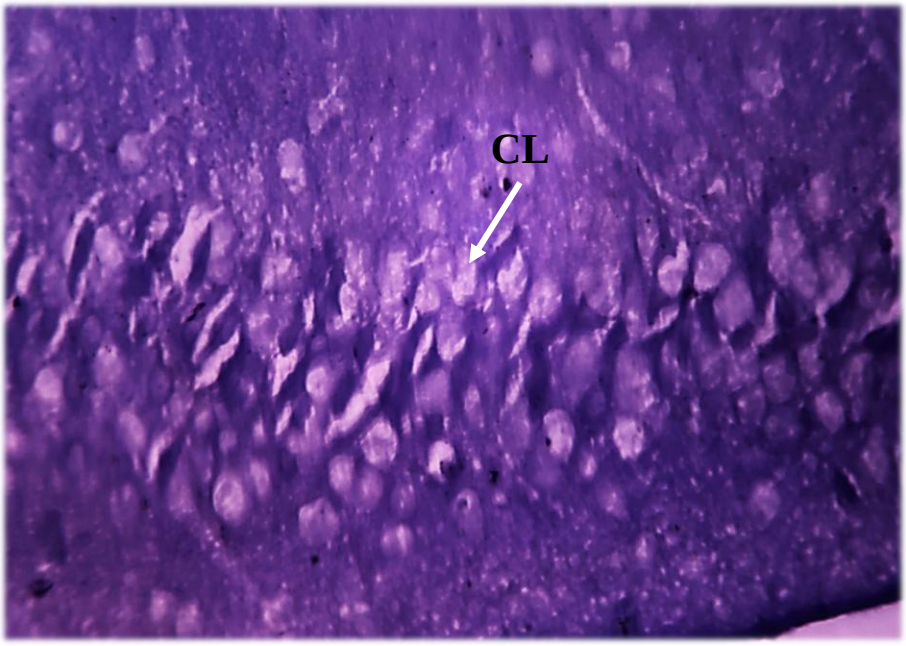


Plate VI: Photomicrograph of the CA3 region of the hippocampus of Wistar rat pups in Group 3 exposed to lead acetate via lactation from dams administered 90mg/kg of lead acetate from PND 1-21 with very weak staining for Nissl substance and chromatolysis. (Cresyl violet stain MagX250).

CL- Chromatolysis

4.4.3 Tomato lectin stain for microglia

Plate VII shows the hippocampusof Wistar rat pups in Group 1 (control group) administered distilled water showing moderatepositive staining of resting microglia and activated microglia cells for tomato lectin stain. The photomicrograph of hippocampus of Wistar rat pups in Group 2 exposed to lead acetate via lactation from dams administered 60mg/kg of lead acetate from PND 1-21 showed presence of more specific binding of tomato lectin with poly N acetyl lactosamine sugar residue present in the cytoplasm and cell membrane of the activated microglia when compared with the control and less presence of activated microglia cells when compared with group 3 as seen in plate VIII. The hippocampus of Wistar rat pups in Group 3 exposed to lead acetate via lactation from dams administered 90mg/kg of lead acetate from PND 1-21 showedmore positive stainingof tomato lectin for the poly N acetyl lactosamine sugar residue present in the cytoplasm of activated microglia when compared to control and group 2 (PlateIX).

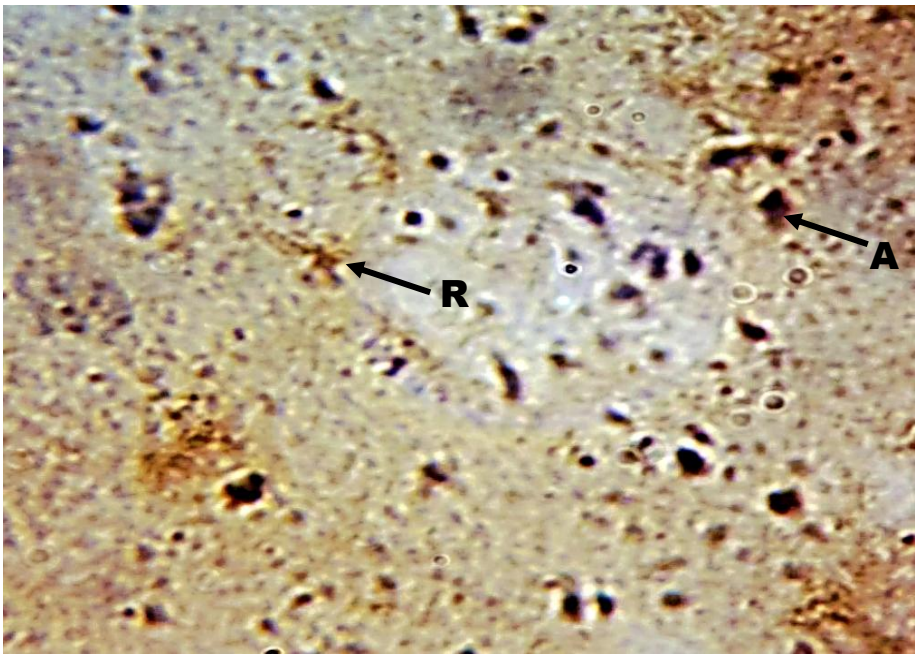


Plate VII: Photomicrograph of the hippocampus of Wistar rat pups in Group 1 (control group) administered distilled water withmoderate positive staining of resting microglia and activated microglia. (Tomato lectin stain MagX1000 oil).

R – Resting microglia
A – Activated microglia

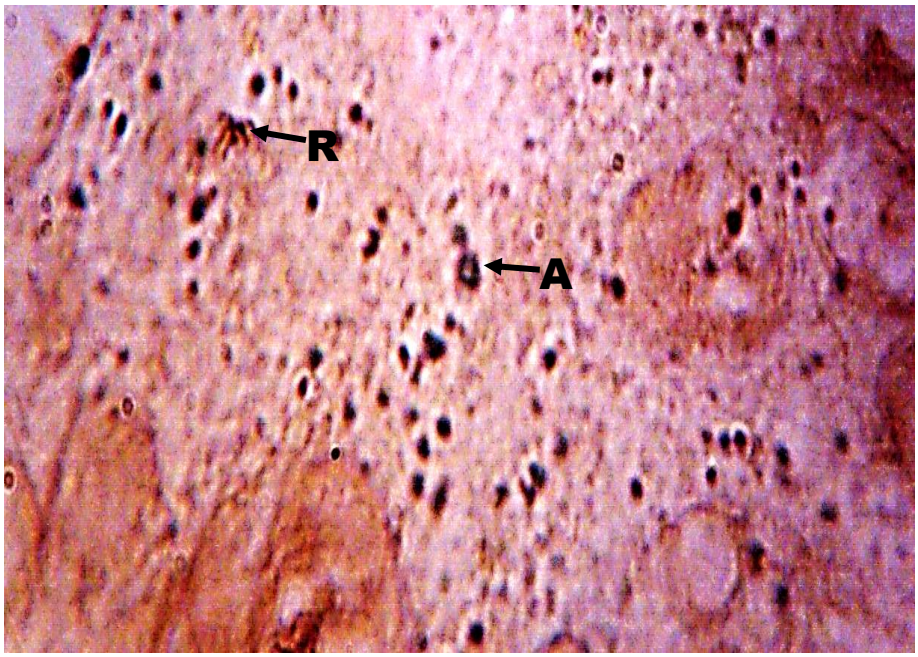


Plate VIII: Photomicrograph of the hippocampus of Wistar rat pups in Group 2 exposed to lead acetate via lactation from dams administered 60mg/kg of lead acetate from PND 1-21 withpresence of more specific binding of tomato lectin with activated microglia cells(Tomato lectin stain MagX1000 oil).

R – Resting microglia
A – Activated microglia

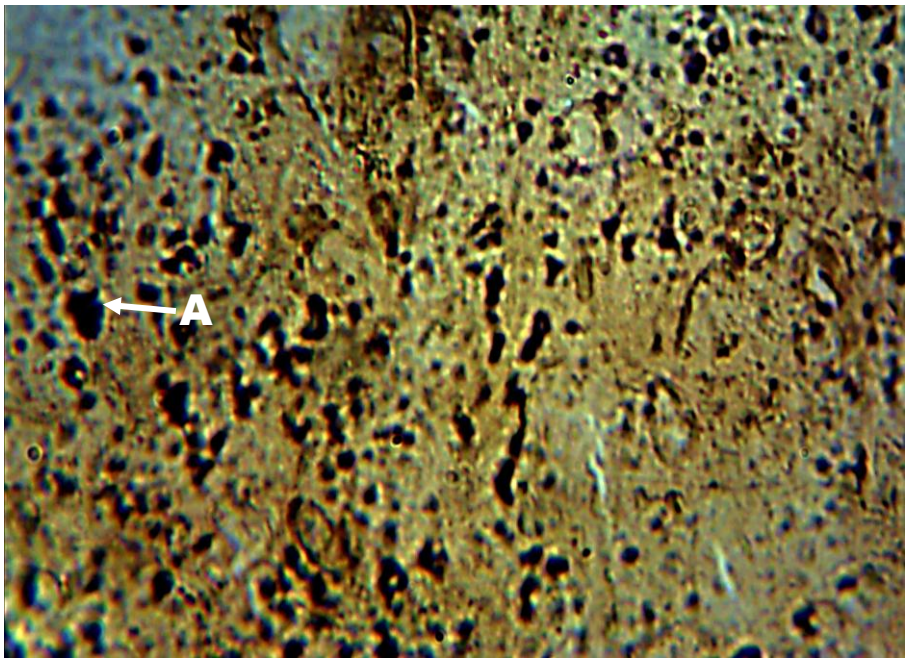


Plate IX: Photomicrograph of the hippocampus of Wistar rat pups in Group 3 exposed to lead acetate via lactation from dams administered 90mg/kg of lead acetate from PND 1-21 with presence of more specific binding of tomato lectin with activated microglia cells(Tomato lectin stain MagX1000 oil).

A – Activated microglia

4.5 Volume of Hippocampus and Number of Activated Microglia Cells

4.5.1 Volume of hippocampus

There was an insignificant ($p>0.05$) decrease in volume ($22.98\pm6.90mm^3$) (coefficient of error (C.E) = 0.086) of hippocampusof pups in group 2 when compared with those of the control ($28.50\pm4.26mm^3$) (C.E = 0.079) and group 3 ($32.04\pm10.52\ mm^3$) (C.E = 0.074) from PND 1-21(Figure 4.4).

4.5.2 Number of Activated Microglia Cells

There was an insignificant ($p>0.05$) increase in number of activated microglia cells in the hippocampus of group 2 ($1.92x10^6\pm6.10x10^5$) (C.E = 0.10) and group 3 ($2.80x10^6\pm1.11x10^6$) (C.E = 0.16) in comparison with the control ($1.22x10^6\pm3.01x10^5$) (C.E=0.18) from PND 1-21 (Figure 4.5).

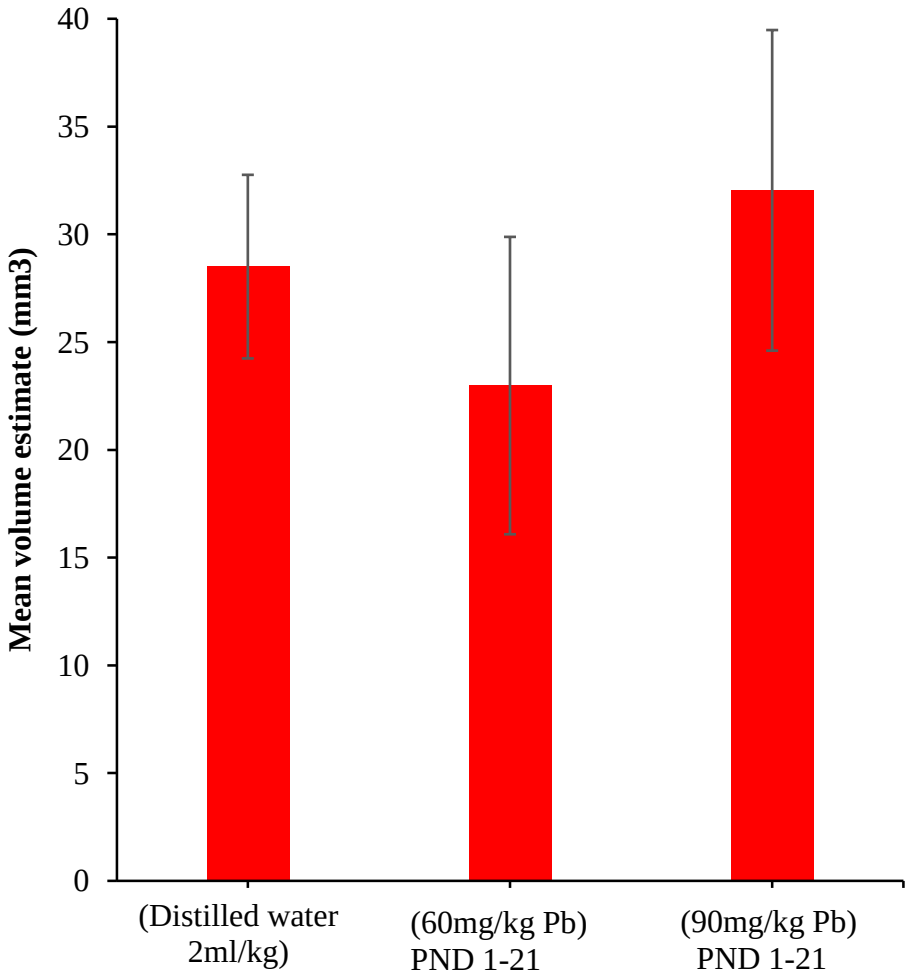


Figure 4.4:Mean volume estimate (mm^3)of hippocampusin lead acetate treated Wistar rat pups. ($p>0.05$).PND - post natal day. n = 2.Pb - Lead

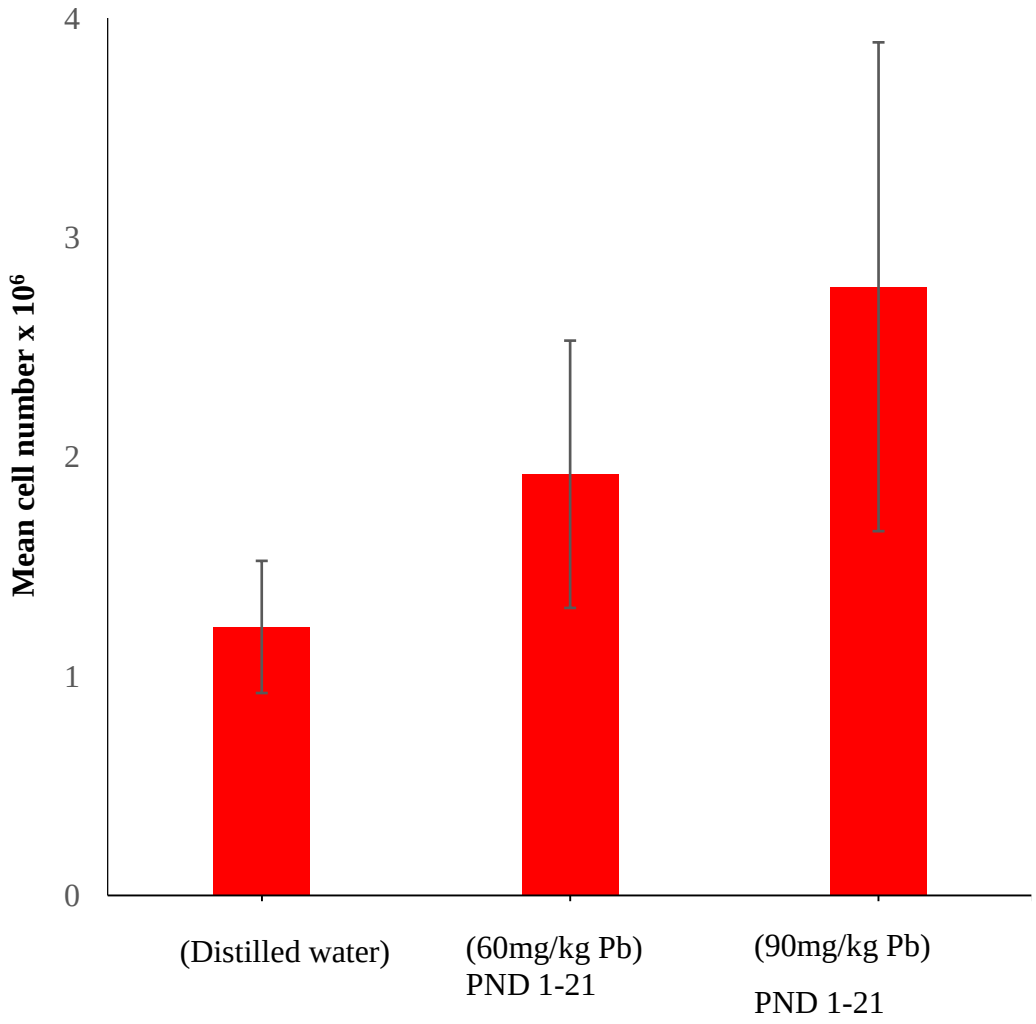


Figure 4.5:Mean number of activated microglia cells in the hippocampusof lead acetate treated Wistar rat pups. ($p>0.05$).PND - post natal day. n = 2.Pb - Lead

CHAPTER FIVE

5.0 DISCUSSION

Previous research suggested that Pb exposure promotes neuroimmune disruption via diverse mechanisms, and possibly chronic microglial activation and microglial proliferation (Kraft and Harry, 2011). A number of studies have revealed that Lead metal causes toxicity in living cells either by ionic mechanism or that of oxidative stress (Flora *et al.,* 2012). Compared to adults, children are more predisposed to its toxicity due the greater absorptive capacity of their gastrointestinal tracts (CDC, 2005). The present study was designed to assess the effects of maternal exposure to different doses of lead acetate during lactation on the hippocampus of developing Wistar rats.

5.1 Body Weight Assessment

In this study a significant decrease in body weight was observed in Wistar rats pups of group 2 and group 3 exposed to lead acetate via lactation from dams administered 60mg/kg and 90mg/kg bwt of lead acetate respectively from PND 1-21 when compared with the control. Wistar rat pups in group 3 exposed to lead acetate via lactation from dams administered 90mg/kg bwt of lead acetate also showed a decrease in body weight when compared to group 2 exposed to lead acetate via lactation from dams treated with 60mg/kg bwt lead acetate from PND 1-21. This suggests that exposure to lead may cause serious loss of body weight in developing infants. The decrease in body weight may be as a result of decline in the consumption of food (anorexia) which is induced by heavy metal consumption. Another possible explanation for the loss of body weight may be the decreased muscle mass and cachexia due to the oxidative stress induced by lead (Zaheer *et al.,* 2013). Aprioku and Siminialayi, (2013) reported significant and time-dependent reductions in body weight after

perinatal exposure to 4 or 8 mg/kg lead acetate respectively. In a related study, weight reductions was also reported in neonates of female rats that received daily injections of 5 mg/kg lead acetate at different stages of pregnancy (Chandra *et al.,* 1983). Ronis *et al.,* (1998) also reported that the treatment of pregnant rats at doses of Pb between 0.05% and 0.45% w/v causes disturbances in pups' body weight at birth, and growth suppression during the pre-puberty and the puberty periods by causing a decrease in the plasma concentration of IGF (Insulin-like growth factor) and the rate of pituitary growth hormone (GH) production. The result from the present study is dissimilar to the findings ofLewis and Pitts, (2004) who reported an insignificant decrease in body weight of Wistar rat pups after 3-week post-natal exposure to lead acetate at (50, or 250 ppm) when compared with the control. The difference observed in the result ofLewis and Pitts, (2004) with that of the present study may be due to the differences in the doses used in the studies.

5.2 Brain Weight and Brain Somatic Index

The findings from the present showed an insignificant decrease in brain weight of Wistar rats pups treated with lead acetate from PND 1-21 when compared with the control. The result from this study is dissimilarto the work of Alyaa *et al.,* (2015) who reported a significant reduction in fetal brain weight of Sprague–Dawley rat pups exposed to lead from dams taking drinking water with 0.5% and 1% lead nitrate for two weeks before conception, and during pregnancy when compared to the control group. Alyaa *et al.,* (2015) stated that lead-induced apoptosis in the cerebellum and possibly in other parts of the nervous system may be the cause for reduced brain weight explained by the increase in Bax/Bcl-2 ratio and caspase 3 expression in the fetal rat cerebella in lead-exposed group indicating up-regulation of mitochondrial apoptosis pathway (Green and Reed, 1998). The reason for dissimilarity in the result of the present study and that of Alyaa *et al.,* (2015) could be due to the difference in

lead compound administered to the rats, the period and duration of administrationas well as the species of the animals used for the experimental study. These finding is also dissimilar to the findings of Corpas *et al.,* (2002) and Acharya *et al.,* (2003) who indicated that testes weights significantly decreased in experimental pups and lead-treated mice exposed to lead perinatally when compared to respective control groups.

The result from this study also showed an insignificant increase in the brain weight of Wistar rat pups in group 3 exposedto lead acetate via lactation from dams administered 90mg/kg bwt of lead acetate in comparison with Wistar rat pups group 2 exposed to lead acetate via lactation from dams that received 60mg/kg of lead acetate from PND 1-21. This is dissimilar to the findings of Nabil *et al.,* (2012) reported a significant increase in the weight of the Liver, Kidney and thyroid gland in male albino rats treated orally with (1/20, 1/40 and 1/60 of the $LD_{50)}$ of lead acetate once in two days for 14weeks. Saeed, (2016) also reported a significant increase in the weight of the Liver, Kidney, Heart and Spleen with increasing concentration of lead in male and female albino rats treated with sublethal doses of lead acetate (0.4, 0.8, and 1.2 mg/kg body weight, respectively) in drinking water for 12 weeks. The dissimilarity in the result from this study and that of Nabil *et al.,* (2012) and Saeed, (2016) could be due to the differences in the duration of exposure to lead.Khalil-Manesh *et al.,* (1992) also reported a significant increase in absolute Kidney weight after lead exposure andattributed accumulation of lead in the form of nuclear inclusion bodies in the proximal tubular cells as the reason for this increase.

The result from the present study also showed a significant increase in brain somatic index ofWistar rat pups in the lead exposed group when compared with the control. From this results it can be deduced that the brain weight of Wistar rat pups exposed to lead acetate via lactation did not reduce as rapidly as their body weight after exposure to lead. That is, the

decrease in body weight observed could be as a result of the decrease in other organs of the body and not necessarily a consequence of decrease in brain weight. This result also suggests that the decrease in body weight observed could be as a result of lead causing irritation of the gastrointestinal tract and disruption of the activity of the lateral hypothalamus, hence reducing food consumption. This is similarto the findings of Kang *et al.,* (2004) who reported significantly higher brain somatic index in high dose lead treated group when compared with the control. An increase in the dry weight of the kidney and liver relative to the body weight was also reported in male weanling rats exposed to lead, which might be because of nutritional disturbances caused by pair feedings (Yagminas *et al.,* 1990).

5.3 Lead Concentration

The mechanism by which lead (Pb) gets into the brain is not clear. One potential mechanism is the disruption of the blood-brain barrier (BBB) by Pb. However, findings on the effects of Pb on the integrity of BBB are inconsistent. Some investigators have reported no effect of Pb on BBB (Michaelson and Bradbury, 1982; Moorhouse *et al.,* 1988), while others have reported significant disruption of BBB by Pb (Terry, 2009; Rai *et al.,* 2010). In the present study it was observed that lead exposure resulted in significant accumulation of lead in the Pb-exposed rat pups when compared with the control. This issimilarto the findings of Rahman *et al.,* (2012) who reported a significant accumulation of lead in the brain of Wistar rat pups that were exposed to 0.2% lead acetate via their dams' drinking water from PND 1 to 21. Du *et al.,* (2015) also reported significantly higher level of lead accumulation in Sprague–Dawley rats exposed to Pb from parturition to PND 30. The finding from the present study is also similar to the work of Hussain *et al.,* (2016) who reported significantly higher lead accumulation on PND 0, 21, 30, 40 and 50 in Wistar rat pups exposed to 0.1% lead acetate

via drinking water after establishing pregnancy until parturition as compared to age matched controls.

Increased accumulation of Pb in lead exposed groups may have been as a result of significant disruption of BBB by Pb (Terry, 2009; Rai *et al.,* 2010). It is also possible that Pb might have been transported into the brain by the divalent metal ion transporter 1 (DMT1), which is localized in astrocytes and neurons in the normal brain (Ong *et al.,* 2006; Wang *et al.,* 2011). Furthermore, the gastrointestinal absorption of metal cations including Pb is much greater during lactation and pre-puberty periods because of (i) nutritional needs of young animals to grow and (ii) the involvement of the intestinal calcium-binding proteins (CaBP) in the transport of Pb, which make them more vulnerable to the deleterious effects of Pb poisoning (Alya *et al.,* 2015).

5.4Oxidative Stress Markers

Superoxide dismutase (SOD) isa metalloprotein and accomplishes its antioxidant functions by enzymatically detoxifying the superoxide radical (O_2^-). Different responses of SOD have been described in animals exposed to Pb including increases, decreases, or no changes in its activity (El-Sokkary *et al.,* 2005) depending on dose, length of treatment, and the target organ. In the present study, SOD levels decreased in all groups that were exposed to lead acetate from PND 1-21 when compared with the control group. The decrease in SOD level suggests that treatment with higher doses of lead (Pb) may lead to further decrease in the SOD level and the anti-oxidant system as a whole, hence jeopardizing the health of the infant as well as resistance to production of free radicals. Lead may have caused a decrease in SOD levels by blocking the sulfhydryl groups of SOD as well as replacing zinc ions which serve as important co-factors for its activity thereby inactivating them (Flora *et al.,* 2012).The findings of the present study is similarto the findings of Sarath *et al.,* (2007) who reported significant

decrease in SOD activity in the hippocampus of all groups treated with lead acetate with highest decrement observed in the group exposed to the highest dosage of 500ppm for 21 days. Ponce-Canchihuamán *et al.,* (2010) also reported a decrease in SOD level after intraperitoneal administration of 25 mg/0.5 mL of lead acetate weekly. Adler *et al.,* (1993) also reported an inhibition of SOD activity by lead in an in vitro study and indicated that this effect of lead can lead to decreased scavenging of reactive oxygen species (ROS) and result in oxidative damage.

Reduced glutathione (GSH) is a tripeptide containing cysteine that has a reactive –SH group with reductive potency. It can act as a non-enzymatic antioxidant by direct interaction of the –SH group with ROS, or it can be involved in the enzymatic detoxification reactions for ROS, as a cofactor or a coenzyme. The result from the present study showed a significant decrease in the level of reduced glutathione in the brain of Wistar rat pups treated with the highest doses of lead acetate postnatally when compared with the control. The result also suggests that exposure to higher doses of lead (Pb) can lead to more decrease in the GSH level and the anti-oxidant system as a whole, hence exposing the infant to oxidative stress. This decrease inGSH levels may be as a result of lead binding to the –SH group of the enzyme thereby inactivatingthem (Ahamed and Siddiqui, 2007).This decrease in the level of GSH supports the decrease in the level of SOD observed in the lead treated group. This could mean that lead may have caused a disruption in the antioxidant system, hence causing oxidative stress.A decrease in GSH levels in the kidneys was also reported by Ponce-Canchihuamán *et al.,* (2010) after intraperitoneal administration of 25 mg/0.5 mL of lead acetate weekly. Günfer *et al.,* (2006) also reported a decrease in serum GSH levels of aluminum Wistar rats administered with aluminium but this was not statistically significant.

Many studies have showed that MDA, the most used end-product of lipid peroxidation, will be increased with Pb treatment (Xia *et al.,* 2010; Lakshmi *et al.,* 2013). The results from this

present study also indicated that lipid peroxidation, determined by measuring the MDA levels, was amplified in the brain of Wistar rat pups exposed to lead postnatally when compared with the control group. From the result, MDA levels increased with increasing concentration of lead acetate. This suggest that the effect of lead on MDA levels is dependent on the dose of lead acetate or heavy metal exposure. The result corroborates with the decrease in anti-oxidant enzyme (SOD and GSH) that was observed and would mean that post-natal exposure of Wistar rat pups to lead via lactation from dams may have caused oxidative stress and may be the reason for the distortion in cytoarchitecture of the CA3 region of the hippocampus that was observed. The result of the study is similarto the findings of Bennet *et al.*,(2007) and Abdel Moneim, (2016) who reported elevated MDA levels in brains and livers of lead acetate treated rats respectively. Alya *et al.,* (2015) also reported MDA amplification in the liver and kidney after exposure to lead (Pb).

The result from this study showed that lead caused oxidative stress by causing a significant decrease in antioxidant enzyme (SOD and GSH) and a significant increase in lipid peroxidation products (MDA). This is similar to the findings of Flora*et al.*,(2012) andWadhwa *et al.,* (2012) who both reported that exposure to lead causes oxidative stress and suggested that this could be the mechanism through which lead causes its neurotoxicity.

5.5 Histological Studies

Results from histopathological examination of tissues is useful in categorizing the type of lesions caused by xenobiotics and is recognized as the most sensitive end point for detecting organ toxicity (Lanning *et al.,* 2002). These results are also useful in providing evidence of acute or chronic effects exposure to toxic substances that may not be detected by other biomarkers (Jadhav *et al.,* 2007). The primary target of low level lead exposure is the central nervous system. The developing brain appears to be especially vulnerable to lead

neurotoxicity. Lead neurotoxicity causes impairment of cognitive abilities and a significant delay in behavioural development, especially in children.

Light microscopic examination of the lead treated groups in this study revealed various degrees of histological changes in the CA3 region of the hippocampus which include presence of necrotic pyramidal cell, cytoplasmic vacuolation, cell loss, presence of degenerating pyramidal cells,, inflammatory cells, clumping of cells, and pyknotic nucleus when compared with the control group suggesting the toxic effects of post-natal lead exposure. These changes were dose dependent, with higher doses causing more damaging effects.

In this study, the necrosis of pyramidal cells in the CA3 region of the hippocampus of Wistar rat pups exposed to lead postnatally via lactation from dams exposed lead acetate may be as a result of oxidative stress due to the depletion of the antioxidant system (SOD and GSH) by post-natal lead exposure as chronic lead exposure have been reported to cause necrosis of hepatocytes due to oxidative stress on these cells by glutathione depletion (El Sayed *et al.,* 2015). Lead induces cell death in the brain, kidney, liver and other organs by stimulating an intrinsic apoptosis pathway (Oberto *et al.,* 1996). Apoptotic alteration might be followed by organelles swelling especially the mitochondria, endoplasmic reticulum and rupture of lysosomes which might lead to amorphous eosinophilic cytoplasm as an initial sign in the sequence of necrosis of neurons before shrinking and dissolution of nuclei, also the leakage of lysosomal hydrolytic enzymes that lead to cell digestion by lytic enzymes (Monte, 2005; Pandey *et al.,* 2008).

The presence of pyknotic nucleus and degenerating cell in the CA3 region of the hippocampus of Wistar rat pups exposed to lead postnatally via lactation from dams exposed lead acetateissimilar to the work of Omayma *et al.,* (2011) who also reported appearance of

some degenerated cells with pyknotic nuclei in the CA1 region of the hippocampus. This was also consistent with the results of Upasani *et al.*,(2001)who attributed these results to the direct effect of lead on the hippocampal neurons. These findings are also analogous with the reports of Belzung and Griebel, (2001), Amal and Mona, (2009) and Abbas, (2015) who stated that administration of different doses of lead caused pyknosis of neurons associated with focal gliosis in addition to focal cerebral haemorrhage.

Post-natal lead exposure also caused cytoplasmic vacuolation in the pyramidal cells in the CA3 region of the hippocampus in lead treated groups. This is similarto the findings of Yusuf *et al.,* (2017) who reported cytoplasmic vacuolations in the molecular layer and Purkinje cell layer of the cerebellum following oral administration of 120mg/kg of lead acetate for 14 days. Mollendorf, (1984) suggested that cytoplasmic vacuolation is mainly a consequence of disturbances in lipid inclusions and fat metabolism. Cytoplasmic vacuolation might also result from disturbance in the oxidative phosphorylation in the mitochondria with suppression of ATP production and failure of the ATP dependent sodium pump at the cell membrane, resulting in accumulation of sodium intracellularly and consequent entry of water into the different cellular compartments which results in cellular swelling (Haouas *et al.,* 2014).

Microglia and astrocytes are two major types of glial cells (inflammatory cells) involved in the regulation of the immune response to pathological processes in the brain (Liu *et al.,* 2011). Functional activation of microglia and astrocytes and the resulting neuroinflammation are associated with infection, autoimmunity, and pathogenesis of neurodegenerative diseases. The increase in inflammatory cells seen in our result may be due to post-natal exposure to lead. Increase in this inflammatory cells can increase the production and release of inflammatory cytokines, enhance generation of reactive oxygen species (ROS), impede antioxidant activity, and result in neuronal injury or neuronal loss in the brain or other parts of the central nervous system (CNS) (Kasten-Jolly *et al.,* 2011; Kumawat, *et al.,* 2014).

Nissl substances are seen as dark spots in an optical microscope. They are dispersed throughout the neuronal perikaryon. At the ultrastructural level, they correspond to 1-4 μm regions abundant with granular endoplasmic reticulum, ribosomes and polysomes. These are the centers of intense protein synthesis used in the transmission of neurotransmitters. After synthesis, proteins are transported along neurites (Fedorenko and Uzdensky, 2010). Cresyl violet stain for Nissl substance in the perikaryon of the pyramidal cells in the CA3 region of the hippocampus of Wistar rat pups exposed to lead postnatally via lactation from dams exposed lead acetate showed weak staining for Nissl substance. These changes were dose dependent, with higher doses causing more negative effects. This is similar with the work of Adekomi *et al.*, (2017) who reported dispersed perinuclear Nissl deposits in cerebral cortex of male Wistar rat exposed to 5 mg/kg Pb-nitrate orally for 21 days. Syed, (2015) and Hamza *et al.,* (2017) also reported loss of Nissl substance from Purkinje cells of the cerebral cortex in Wistar rats exposed to varying doses of lead. The degeneration of the Nissl substance may lead to inadequate synthesis of neurotransmitters. The mossy fibres of the granule cell in the dentate gyrusconducts neurotransmitter from the perikaryon of the granule cells to the dendrite of the pyramidal cells.Degeneration of the Nissl bodies decreases synthesis of neurotransmitter which in turn affects the transmission of impulses to the pyramidal cells of the CA3 region of the hippocampuswhich in turn leads to anterograde and retrograde amnesia (Ekundina *et al.,* 2016). These neurodegenerative changes could invariably impair the activities of the hippocampus in memory formation, learning, storage and retrieval of information. The present findings are similar to previous reports of Khaled *et al.,* (2014), who observed neuronal damage in cerebral cortex, hippocampus and cerebellum, with neurodegeneration of CA1 and CA3 regions of Sprague Dawley rats exposed to lead acetate.

Given the potential neurodegenerative effects of disrupted neuroimmune function, we also examined the effect of post-natal lead exposure on the volume of the hippocampus. From the

present studyan insignificant increase in the volume of the hippocampus was observed inWistar rat pups exposed to lead postnatally via lactation from dams exposed 90mg/kg bwt of lead acetatewhen compared with the control. We also observed an insignificant decrease in the hippocampus volume of Wistar rat pups exposed to lead postnatally via lactation from dams exposed 60mg/kg bwt of lead acetatewhen compared with the control andWistar rat pups exposed to lead postnatally via lactation from dams exposed 90mg/kg bwt of lead acetate. This disagrees with the work of Sobin *et al.,* (2013) who reported that when compared with controls, the dentate gyrus volume in young mice exposed to 30ppm and 330ppm lead acetate respectively for 28 days was significantly decreased, but exposure groups did not differ significantly. This also disagrees with the work of Stewart*et al.,* (2006) who reported that high tibia leadwas associatedwith reduced total brain volume, lower volume of gray matter in the insula and cingulum, and diminished white matter volume in the parietal lobes of 532 lead workers. The reason for the increase in volume observed may be due to the increase in proliferation of microglia cells as well as other glia cells in the hippocampus of the Wistar rats as a result of post-natal lead exposure (Verstraeten*et al.,* 2008).

Microgliaare glial cell found throughout the central nervous system (CNS) (Ginhoux *et al.,* 2013). They are the resident macrophages of the brain and spinal cord with important roles in development, homeostasis, disease, and injury (Aguzzi *et al.,* 2013). They are associated with the blood brain barrier (BBB) and are among the first cells to respond to threats to the brain (Midori *et al.,* 2006). They provides the first line of defence against any disease or insult and display enormous structural and functional plasticity which may include; proliferation, chemotaxis, morphological alterations, and generation of immunomodulatory molecules. Paolicelli *et al.,* (2011) suggested that activated microglia surround the regions undergoing developmental synapse turnover, and remove the unnecessary synapses in mice.Microglia are

also believed to be involved in regulation of neuronal proliferation and differentiation during development (Farinas *et al.,* 2002).They also help in regulation of long-term potentiation (LTP) and tuning of synaptic strength by releasing various soluble molecules responsible for regulating learning and memory and augmentation of N-methyl-D-aspartate (NMDA)-mediated LTP responses. (Ben Achour and Pascual, 2010; Kettenmann *et al.,* 2011). Although one study suggested that the microglia released IL-8 is involved in the inhibition of hippocampal LTP (Xiong*et al.,* 2003).

Microglial cells areimportant in physiological or pathophysiological functions (Yin*et al.,* 2008; Schlegelmilch*et al.*,2011). Treatment with some neurotoxic substance such as Lipopolysaccharide (LPS) impairs the rat learning and memory (Tanaka*et al.,* 2006) and induces the microglia activation (Espinosa-Oliva*et al.,* 2009). Lead is also a neurotoxic substance, therefore, it is normal to speculate that microglia may play an important role in the Pb exposure induced learning and memory decline.

With regard to the number of activatedmicroglia cells, counter to our hypothesis, as compared with control there was an insignificant increase in the number of activated microglia cell of Wistar rat pups exposed to lead postnatally via lactation from dams exposed 60mg/kg bwt and 90mg/kg respectively from PND 1 - PND21. This differs from the work of Liu *et al.,* (2012) who reported a significant increase in presence of activated microglia cells when compared to resting microglia in the hippocampus of weaning Sprague-Dawley rats (SD-rats) administered lead acetate for eight weeks. The result from the present study also differs from the work of Liu *et al.,* (2015) who reported a significant increase in the number of microglial cells in the hippocampus of young mice treated with 15mg/kg of lead intraperitoneally for 3days when comparedwith the control. The result of this study is also differs from that of Sobin *et al.,* (2013) who reporteda significant decrease in microglia mean

cell body number in the dentate gyrus of young mice after post-natal exposure to lead at 30 ppm 330ppm respectively when compared to the control.

There are several reasons for dissimilarity in the result of the present study and that of Liu *et al.,* and Liu *et al.,* (2015) and the insignificant increase in number of activated microglia cells observed. Tomato lectin binds specifically to the Poly N acetyl lactosamine present in the cytoplasm of the microglia, lead may have caused disruption of this carbohydrate within the cytoplasm preventing proper binding(which in turn could disable normal microglial cell function). Another explanation may be due to proliferation mechanisms. Studies have suggested that the P2X7 receptor is critical for microglial proliferation during activation (Monif *et al.,* 2010). It is possible that lead may have caused disruption of this P2X7 receptor therefore inhibiting rapid proliferation. The effects of Pb on microglia progenitors could also be a factor. Microglia are derived from hematopoietic stem cells in bone marrow. Some of these stem cells differentiate as monocytes and further differentiate as microglia in the brain (Ritter *et al.,* 2006). Pb is sequestered in bone marrow andits presence may have disrupted critical replenishment of the hematopoietic daughter cell pool, thus reducing the migration of adequate progenitor cell numbers to the hippocampus. Another explanation could be that the amount of lead that crossed the breast milk during lactation and in turn the BBB was not able to cause significant proliferation and prolonged activation of microglia cells. The duration of exposure of Wistar rat pups to lead acetate postnatally may be too short to trigger significant microglia activation.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The results obtained from the present study revealedthat post-natal exposure of Wistar rat pups to lead via lactation from dams administered lead acetate orally from PND 1-21 caused:

i. a significant decrease in body weight, significant increase inbrain somatic index and an insignificant decrease inthe brain weight.
ii. a significant accumulation of lead in the brain.
iii. oxidative stress in the brain by causing a significant decrease in antioxidant enzymes (SOD and GSH) level and a significant increase in MDA levels.
iv. distortion of the cytoarchitecture of the CA3 region of the hippocampus.
v. an insignificant increase in the volume of the hippocampus.
vi. an insignificantincrease in the number of activated microglia cells in the hippocampus.

6.2 Conclusion

The effect of maternal exposure to different doses of lead acetate during lactation on the hippocampus of developing Wistar rats was evaluated in this study.With respect to the findings of the present study,it can be inferred that exposure to increasing doses of lead acetate from post-natal day 1-21 did not significantly trigger microglia activation but caused distortion in the cytoarchitecture of the CA3 region of the hippocampus of Wistar rat pups.

6.3Contribution to Knowledge

This study established thatWistar rat pups exposed to lead via lactation from dams administered lead acetate orally at a dose of 60 mg/kg and 90 mg/kg bwt respectively from PND 1-21 caused:

i. significant increase ($p<0.05$) in lead accumulation in brain tissues with values of (0.04±0.01ppm) and (0.11±0.03ppm) respectively when compared with the control (-0.05±0.02ppm).
ii. significant increase ($p<0.05$)in MDA levels(25.20±0.80nmol/mg protein) and (30.23±1.96nmol/mg protein) respectively when compared with the control (23.07±0.63nmol/mg protein),significant decrease ($p<0.05$) in SOD levels(17.90±0.25 U/ml) and (16.50±0.46 U/ml) respectively when compared with the control (20.60±0.56 U/ml) anda significant decrease ($p<0.05$) in reduced glutathione (GSH) in Wistar rat pups exposed to lead acetate via lactation from dams administered lead acetate orally at a dose 90 mg/kg bwt(11.40±0.99 ug/ml) respectively when compared with the control (16.50±1.48 ug/ml).
iii. an insignificant increase ($p>0.05$) in the number of activated microglia cells with values of $1.92x10^6±6.10x10^5$ (CE = 0.10)and $2.80x10^6±1.11x10^6$(CE = 0.16) respectively when compared with the control$1.22x10^6±3.01x10^5$ (CE = 0.18).

6.4 Recommendations

The present study recommends:

i. that lactating mothers should avoid exposure to lead.

ii. further studies on the effect of higher doses and longer duration of post-natal lead exposure on the developing brain.

iii. further studies on the effect of post-natal lead exposure on learning and memory using neurobehavioural test.

iv. that future studies could also examine the effects of brain Pb after post-natal exposure on expression of P2X7 since studies have suggested that the P2X7 receptor is critical for microglial proliferation during activation.

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APPENDIX I

ETHICAL APPROVAL

AHMADU BELLO UNIVERSITY, ZARIA
DIRECTORATE OF ACADEMIC PLANNING & MONITORING
Vice Chancellor: Prof. Ibrahim Garba, B.Sc. (Hons) Geology, M.Sc. (Mineral Exploration) A.R.C., Ph.D Geology (London), D.I.C., FNMGS
Director: Prof. M.B. Ibiyeye, B.Sc. (Hons) Botany (ABU), M.Sc. Plant Breeding (U.K.), Ph.D Agriculture (University of Reading, U.K.), MASSN, MIBN
Tel. Zaria + 234-069-551912 Fax. 234 (069) 550022 Telegram: UNIBELLO Zaria e-mail: dapm@abu.edu.ng

Appl No.: ABUCAUC/2018/Human Physiology/047
Approval No: ABUCAUC/2018/047

3rd October, 2018

Prof. S.S. Adebisi
Department of Human Anatomy,
Faculty of Basic Medical Sciences,
College of Health Sciences,
Ahmadu Bello University,
Zaria.

Dear Sir,

APPROVAL OF RESEARCH STUDY 'ASSESSMENT OF THE EFFECT OF POST-NATAL LEAD EXPOSURE ON MICROGLIA ACTIVATION IN THE HIPPOCAMPUS OF DEVELOPING WISTAR RATS'

This is to convey the approval of the ABUCAUC to you for the aforestated study domiciled in the Department of Human Anatomy. The approval is predicated on the assumption that you shall maintain and care for the Experimental Animals as approved after the visitation of the Committee.

Monitoring of the Research by spot checks, invitations or any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCAUC Page on the University's website.
Note upon completion of the research, ethical clearance certificate will be issued.

U.D. Abdullahi
For: Chairman, ABUCAUC.

Cc. Director, DAPM
Director, IC &ICT
Provost, College of Health Sciences
Dean, Faculty of Basic Medical Sciences,
HOD, Human Anatomy,
Prof. Aliyu Mohammed

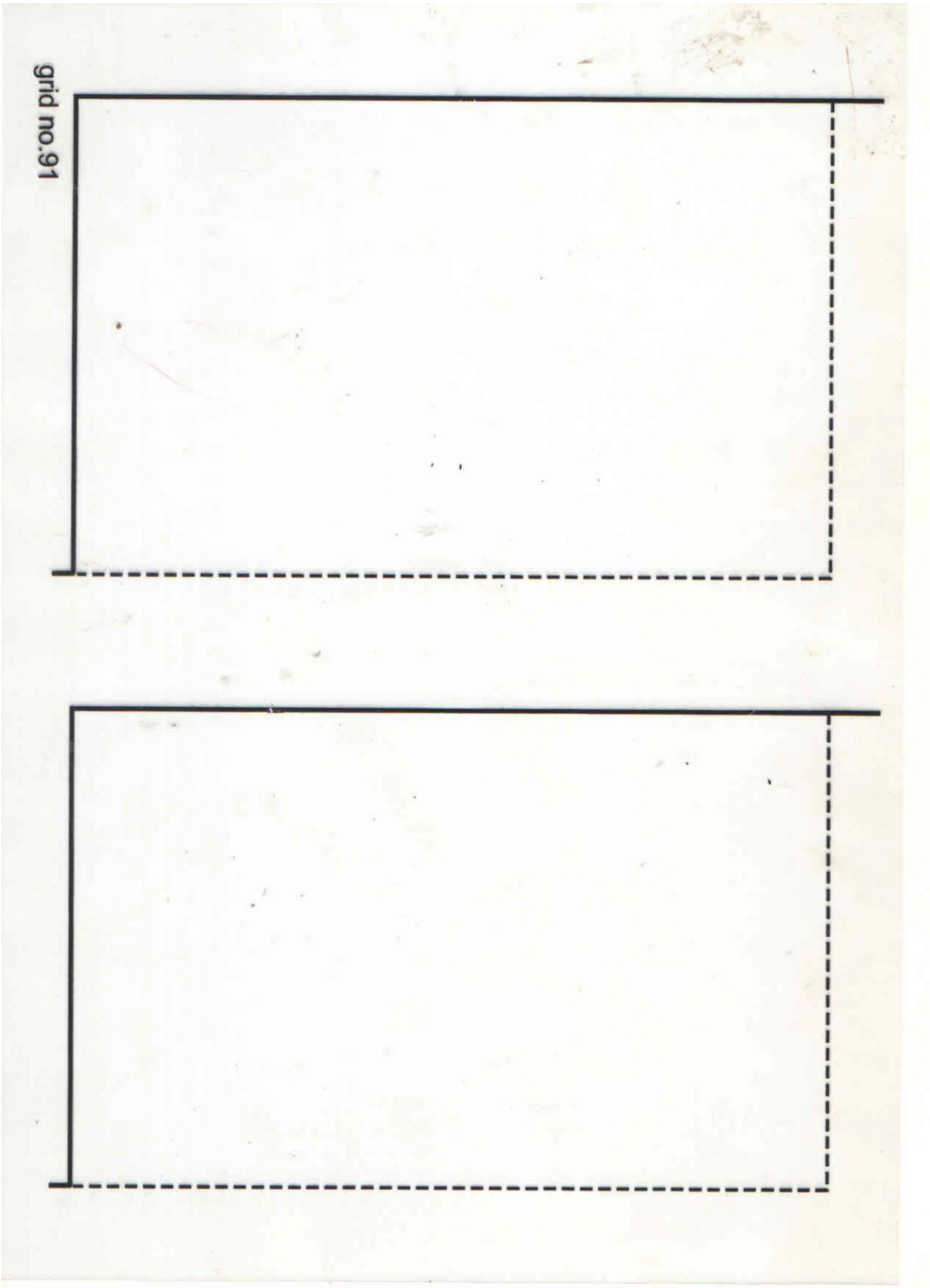
APPENDIX II

COUNTING GRID



APPENDIX III

COUNTING FRAME


grid no.91