EFFECT OF *Trypanosoma brucei brucei* INFECTION ON SPATIAL MEMORY, HISTOLOGY AND EXPRESSION OF CALBINDIN-D28K IN HIPPOCAMPUS OF WISTAR RATS.

BY

DANIEL, RICHARD MENDUWAS

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Daniel, Richard Menduwas

(B.Sc Human Anatomy, 2010)

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS OF SCIENCE (M.Sc) DEGREE IN HUMAN ANATOMY

DEPARTMANT OF HUMAN ANATOMY

FACULTY OF MEDICINE

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MARCH, 2015.

DECLARATION

I, Daniel, Richard Menduwas, hereby declare that the work in the thesis titled "Effect of *Trypanosoma brucei brucei* infection on spatial memory, histology and expression of calbindin-d28k in hippocampus of wistar rats." has been performed by me in the Department of Human Anatomy under the supervision of Dr. (Mrs.) H.O. J. N. Alawa and Dr. W.O. Hamman.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another degree or diploma at any university.

_____ _____

DANIEL, RICHARD MENDUWAS Date

Department of Human Anatomy

Faculty of medicine

Ahmadu Bello University, Zaria.

CERTIFICATION

This thesis titled "Effect of Trypanosoma brucei brucei infection on spatial memory, histology and expression of calbindin-d28k in hippocampus of wistar rats" meets the requirement governing the award of the degree of Master of Science (Human Anatomy) in Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

Dr. (Mrs) J. N. ALAWA (*Ph.D*)	_____	_____
Chairman, Supervisory Committee	Signature	Date
Department of Human Anatomy,		
Ahmadu Bello University, Zaria.		
Dr. W. O. HAMMAN (*Ph.D*)	_____	_____
Member, Supervisory Committee	Signature	Date
Department of Anatomy,		
Ahmadu Bello University, Zaria.		
Prof. S. S. ADEBISI	_____	_____
Head of Department	Signature	Date
Department of Human Anatomy,		
Ahmadu Bello University, Zaria.		
Prof. ALHASSAN ZOAKA	_____	_____
Dean, School of Postgraduate Studies	Signature	Date
Ahmadu Bello University, Zaria		

DEDICATION

THIS THESIS IS DEDICATED

to

MY LOVING MOTHER AND LATE FATHER

And to

MY DEARLY BELOVED BROTHER

And to

MY DEARLY BELOVED SISTERS

And to

EVERYBODY SUFFERING FROM TRYPANOSOMIASIS DISEASES IN AFRICA

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to the success of this work which time and space will not permit me to mention; I say, I appreciate you all!

ABSTRACT

Trypanosomiasis, a diseases in the Sub-saharan Africa; its neurodegeneration phase of is yet to be understood. This study was designed to investigate the effect of *Trypanosoma brucei brucei* on spatial memory, histology and expression of *calbindin-d28k* in hippocampus of wistar rats. Thirty adult (male) Wistar rats were categorized into two groups, control(six rats) and infected (24 rats). Group 2 was divided into 4 subgroups (6 rats/ subgroup). Group 1 served as control, group 2 was inoculated with $1x10^6$ innoculum dose of the parasite. Parasitaemia was studied using wet blood smear preparation. Morphological observations were made using weighting scale, Vanier caliper, and digimizer 2.2. Blood parameters were studied using automated hematological analyser. Histological and histomorphometrical studies were done using H and E, toluidine blue and. Expression of calbindin d28k was observed using the immunohistochemistry study, the antigen/antibody technique. Spartial memory and learning was studied using Morris water maze method and latency time to find the hidden platform was recorded. These were done after 3days for group 2a, 7days for 2b, 14days for group 2c, 21days for group 2d and the control group. Innoculation of the parasite, over time, significantly induced parasitaemia; significantly affected blood parameters(leucopenia, anemia, lymphopaenia and neutropaenia); significantly reduced body weight, neuronal cell count and diameter; it caused distortion of the laminated hippocampal arraingement; it reduced the expression of *calbindin d28k*. Affected learning and memory by decreasing latency time over the research period.In conclusion, *Trypanosoma brucei brucei* infection has deleterious effects on body, blood, hippocampus, and expression of *calbindin d28k*, which was reflected in the decrease in spartial memory and learning of the Wistar rats.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BRD	Breadth
BrW	Brain Weight
BW	Body weight
CA	Cornus Amorni
CTR	Control
H and E	Haematoxylene and Eosine
HGB	Haemoglobin
kg	kilogram
Lym	Lymphocyte
mg	milligram
ml	milliliters
Neut	Neutrophil
PI	Post Infection
PRI	Pre-Infection
RBC	Red blood cells
TBB	*Trypanosoma brucei brucei*
TLC	Thin layer chromatography
TPC	Total phenolic content
UNICEF	United Nations Children's Fund
WBC	White blood cells
WID	Width
WHO	World Health Organisation

CHAPTER ONE

1.0 Introduction

1.1 Background Information

Trypanosomiasis is a parasitic disease of people and animals, caused by protozoa of the Trypanosoma genus and transmitted by the tsetse fly (WHO,2006). It ranks high in importance amongst protozoan infections of animals and man. It occurs in tropical and subtropical regions of the world and affects different species of animals like horse, camel, dog, cattle, sheep and goats (Boid, 2011). The disease is also called sleeping sickness, Human African Trypanosomiasis (HAT) or Congo trypanosomiasis (Robinson,2009).

1.1.1 Incidence in Africa

162,877 cases have been reported in africa between 2001 to 2011, according to WHO in 2012, with the distribution shown in the table below:

Table 1.1: Incidence of trypanosomiasis in Africa from 2001 to 2011.

Country	**Percentage(%)**
DRC	58.27
Angola	11.18
Sudan	8.85
Uganda	1.68
Chad	2.04
Cameroon	0.12
Nigeria	0.08
Benin, Burkina faso, Ghana and Mali	0

1.2 Symptoms

African trypanosomiasis symptoms occurs in two stages:

Haemolymphatic Phase

Invasion of the circulatory and lymphatic systems by the parasite is associated with severe swelling of the lymph nodes, often to treamendous sizes. This is characterised by:

- Fever
- Headaches
- Joint pains and
- Itching.

If left untretad, the disease overcomes the host's defences and causes more extensive damage, broadening symptoms to include:

- Anemia
- Endocrine disfunction
- Cardiac disfunction and
- Kidney disfunction.

Neurologic Phase

The neurological phase begins when the parasite invades the CNS by passing through the blood-brain barrier. The term 'sleeping sickness' comes from the symptoms of the neurological phase. The symptoms include confusion, reduced coordination and disruption of the sleep cycle, with bouts of fatigue punctuated with manic periods, leading to daytime slumber and night-time insomnia. Without treatment, the disease is invariably fatal, with progressive mental detoriation leading to coma and death. Damage caused in the neurological phase is irreversible (New Vision,2008).

1.3 Aims and Objectives of the Study

Aims:

To investigate the effect of *T. brucei brucei* infection on spartial memory, histology and expression of calbindin-D28k neurons of hippocampus in Wistar rats.

Objectives:

i. To determine the effect of T. brucei brucei on the spartial memory and learning using Morris water maze test.

ii. To determine the extent of neurodegenerative damage caused by *T. brucei brucei* in hippocampus using routine histological stains (H & E) and special stains and histomorphometric analysis.

iii. To compare the expression of calbindin-D28k in neurons of hippocampus in rats infected or uninfected with *Trypanosoma brucei brucei* using immunohistochemistry.

1.4 Significances of the Study

Results of this study could help in the understanding of the mechanism involved in the neurologic phase of trypanosoma infections and exploited in the development of drugs.

1.5 Research Hypothesis

T. brucei brucei infection will affect the spartial memory, histology, and expression of calbindin d28k in the hippocampus of Wistar rats.

CHAPTER TWO

2.0 Literature Review

2.1 Trypanosomiasis

Human African trypanosomiasis (HAT or sleeping sickness) has been claimed to be more deadly than any other vector-borne disease such as malaria, because death is inevitable if a patient is not treated (Kennedy, 2004). In spite of the existence of a huge body of research findings on African trypanosomosis (trypanosomiasis), the disease has continued to wreak havoc on human and animal lives with consequent effects on the fragile economy of countries of Tropical Africa (Kristjanson et al., 1999; Bourn *et al*., 2005). Variable disorders occur sequel to trypanosome infection in animals. An in-depth knowledge of mechanisms of their development is pivotal to search for and identification of molecular targets, which could be exploited in evolution of therapeutic approaches, especially, in this cutting edge period of research in molecular medicine and biotechnology. Drug regimen are cumbersome in addition to being expensive (Kioy and Mattock, 2005; Moore, 2005). The search for new drugs and formulations that are safe and effective against both the early and late stages of the diseases is recommended (Jannin and Cattand, 2004; Chibale, 2005; Pink *et al*., 2005).

2.2 History

The condition has been present in Africa since at least the 14th century, and probably for thousands of years before then. Because of a lack of travel between indigenous people, sleeping sickness in humans had been limited to isolated pockets. This

changed once Arab slave traders entered central Africa from the east, following the Congo River, bringing parasites along. Gambian sleeping sickness travelled up the Congo River, then further eastwards. In 1901, a devastating epidemic erupted in Uganda, killing more than 250,000 people (Fèvre *et al*.,2004).

The causative agent and vector were identified in 1903 by David Bruce, and the differentiation between the subspecies of the protozoa made in 1910. The first effective treatment, atoxyl, an arsenic-based drug developed by Paul Ehrlich and Kiyoshi Shiga, was introduced in 1910, but blindness was a serious side effect.

In 1986, it was estimated that some 70million people lived in areas where the disease transmission could take place. In 1998, almost 40,000 cases were reported, but estimates were that 300,000 cases were undiagnosed and therefore untreated (WHO, 2006).

Four major epidemics have occurred in recent history: one from 1896–1906 primarily in Uganda and the Congo Basin, two epidemics in 1920 and 1970 in several African countries, and a recent 2008 epidemic in Uganda (Uganda, 2008).

Sleeping sickness was the first or second greatest cause of mortality in those communities, ahead of even HIV/AIDS. By 2005,surveillance was reinforced and the number of new cases reported on the continent was reduced; between 1998 and 2004, the number of actual cases was between 50,000 and 70,000 (WHO, 2006).

2.3 Transmission

Trypanosomes are transmitted by the tsetse fly as a vector, species of trypanosomes develop and multiply in the midgut initially, before onward migration to the mouthparts; infective metacyclics develop in the proboscis for T. congolense (subgenus Nannomonas)

and in the salivary glands for T. brucei (subgenus Trypanozoon). The metacyclic form of the parasite are found attached to the labrum of the proboscis and in the saliva. The vector deposites the parasites into the blood stream of animals and humans via bites.

Tsetse fly transmit trypanosomes in two ways, mechanical and biological transmission;

1) Mechanical transmission involves the direct transmission of the same individual trypanosomes taken from an infected host into an uninfected host. The name mechanical reflects the similarity of this mode of transmission to mechanical injection with a syringe. Mechanical transmission requires that tsetse feed on an infected host and acquire trypanosomes in the bloodmeal, and then, within a relatively short period, for tsetse to feed on an uninfected host and regurgitate some of the infected blood from the first bloodmeal into the tissue of the uninfected animal. This type of transmission occurs most frequently when tsetse are interrupted during a bloodmeal and attempt to satiate themselves with another meal. Other flies, such as horse-flies, also can cause mechanical transmission of trypanosomes (Cherenet *et al*.,2004).
2) Biological transmission requires a period of incubation of the trypanosomes within the tsetse host. The term biological is used because trypanosomes must reproduce through several generations inside the tsetse host during the period of incubation, which requires extreme adaptation of the trypanosomes to their tsetse host. In this mode of transmission, trypanosomes reproduce through several generations, changing in morphology at certain periods. This mode of transmission also includes the sexual phase of the trypanosomes. Tsetse are believed to be more likely to become infected by trypanosomes during their first few bloodmeals. Tsetse infected by trypanosomes are thought to remain infected for

the remainder of their lives. Because of the adaptations required for biological transmission, trypanosomes transmitted biologically by tsetse cannot be transmitted in this manner by other insects (Cherenet *et al*.,2004).

The relative importance of these two modes of transmission for the propagation of tsetse-vectored trypanosomiasis is not yet well understood. However, since the sexual phase of the trypanosome lifecycle occurs within the tsetse host, biological transmission is a required step in the life cycle of the tsetse vectored trypanosomes (Cherenet *et al*.,2004). The entire life cycle of the fly takes about three weeks. The tsetse vectored trypanosomiasis affect various vertebrate species including humans, antelopes, bovine cattle, camels, horses, sheep, goats, and pigs. These diseases are caused by several different trypanosome species that may also survive in wild animals such as crocodiles and monitor lizards. The diseases have different distributions across the African continent and are therefore transmitted by different species of tsetse fly (Hunt, 2004).

2.3.1 Vector

Tsetse, sometimes spelled tzetze and also known as tik-tik flies, are large biting flies that inhabit much of mid-continental Africa between the Sahara and the Kalahari deserts(Rogers et al., 1996). They live by feeding on the blood of vertebrate animals and are the primary biological vectors of Wuchereria bancrofti, which cause Elephantiasis, and trypanosomes, which cause human sleeping sickness and animal trypanosomiasis, also known as nagana. Tsetse have been extensively studied because of their disease transmission. These flies are multivoltine, typically producing about four generations yearly, and up to 31 generations total over their entire lifespan (Cockerell, 1997).

Fossilized tsetse have been recovered from the Florissant Fossil Beds in Colorado, laid down some 34 million years ago (Cockerell, 1997). There are 23 species of tsetse flies. Diseases transmitted by tsetse flies kill 250,000–300,000 people per year (WHO, 2006).

2.3.2 Morphology of Tsetse Fly

Tsetse fly can be seen as independent individuals in two forms: as third instar larvae, and as adults. They first become separate from their mothers during the third larval instar, during which they have the typical appearance of maggots. However, this life stage is short, lasting at most a few hours, and is almost never observed outside of the laboratory.

Tsetse next become puparia: small, hard shelled, oblongs with two distinctive, small, dark lobes at one end. Tsetse puparia are under 1.0 cm long (Jordan, 2006). Within the puparial shell, tsetse complete the last two larval instars and the pupal stage.

Tsetse then emerge as adult flies. Tsetse adults are relatively large flies, with lengths of ½–1½ cm (Jordan, 2006), and have a recognizable shape or bauplan which makes them easy to distinguish from other flies. Tsetse have large heads, distinctly separated eyes, and unusual antennae. The tsetse thorax is quite large, while the abdomen is wide rather than elongated and shorter than the wings.

Tsetse fold their wings completely when they are resting so that one wing rests directly on top of the other over their abdomen. Tsetse also have a long proboscis, which extends directly forward and is attached by a distinct bulb to the bottom of their head.

Like all other insects, Tsetse flies have an adult body comprising three visibly distinct parts: the head, the thorax and the abdomen:



(Jordan, 2006)

Figure 2.1: Tsetse fly (*Glossina mortistan*).

The head has large eyes, distinctly separated on each side, and a distinct, forward-pointing proboscis attached underneath by a large bulb. The thorax is large, made of three fused segments. Three pairs of legs are attached to the thorax, as are two wings and two halteres. The abdomen is short but wide and changes dramatically in volume during feeding (Gouteux, 1997).

Most tsetse flies are physically very tough. Houseflies are easily killed with a fly-swatter but it takes a great deal of effort to crush a tsetse fly.

The internal anatomy of tsetse is fairly typical of the insects. The crop is large enough to accommodate a huge increase in size during the bloodmeal since tsetse can take a bloodmeal weighing as much as themselves. The reproductive tract of adult females

includes a uterus which can become large enough to hold the third instar larva at the end of each pregnancy (Gouteux, 1997).

2.3.3 Life cycle of Tsetse fly

Tsetse have an unusual life cycle which may be due to the richness of their food source. Female tsetse only fertilize one egg at a time and retain each egg within their uterus to have the offspring develop internally during the first larval stages, a strategy called adenotrophic viviparity. During this time, the female feeds the developing offspring with a milky substance secreted by a modified gland in the uterus. In the third larval stage, the tsetse larva finally leave the uterus and begin their independent life. However, the newly independent tsetse larva simply crawls into the ground, and forms a hard outer shell called the puparial case, in which it completes its morphological transformation into an adult fly. This lifestage has a variable duration, generally twenty to thirty days, and the larva must rely on stored resources during this time. The importance of the richness of blood to this development can be seen since all tsetse development before it emerges from the puparial case as a full adult occurs without feeding, based only on nutritional resources provided by the female parent. The female must get enough energy for her needs, for the needs of her developing offspring, and to store the resources which her offspring will require until it emerges as an adult (Gouteux, 1997).

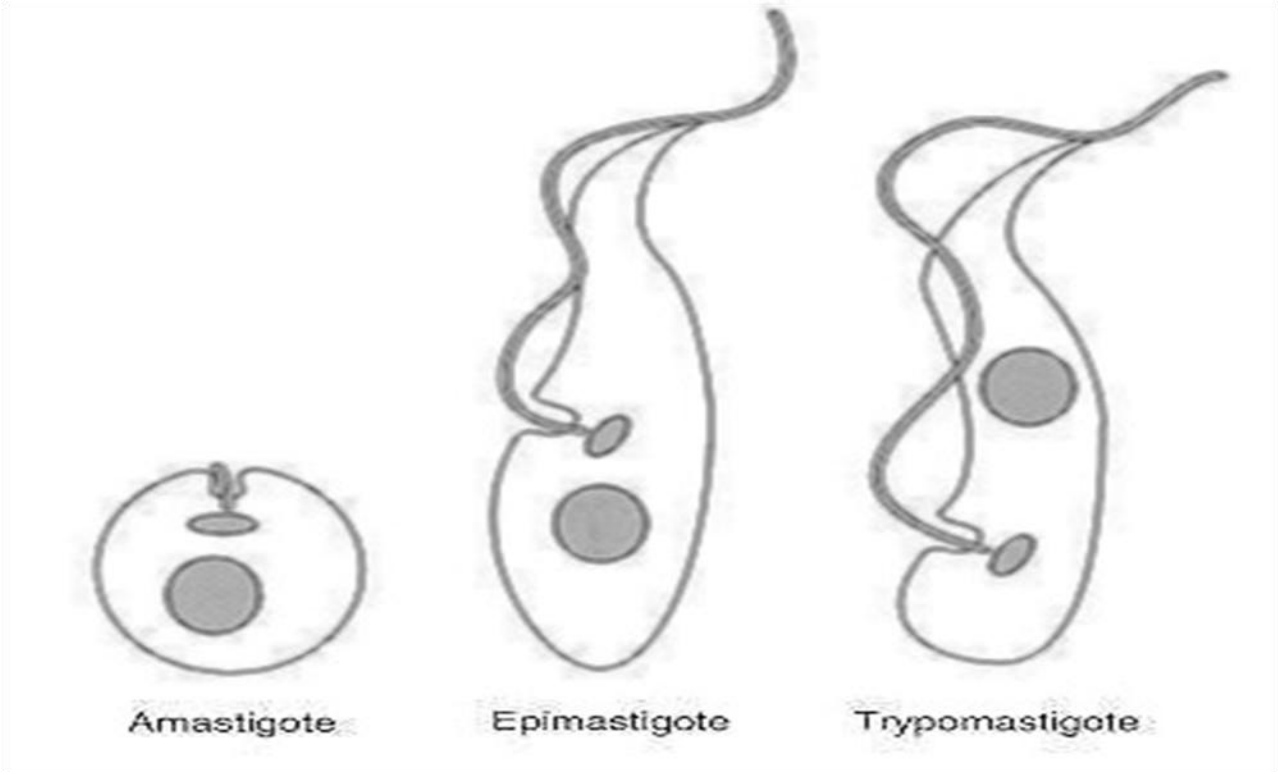
Technically these insects undergo the standard development process of insects which comprises oocyte formation, ovulation and fertilization, development of the egg, five larval stages, a pupal stage, and the emergence and maturation of the adult.

2.4 Parasites

Trypanosomes are unicellular protozoans with a single flagellum that contains microtubules in the 9+2 arrangement typical of other flagella. At the base of the flagellum is the kinetoplast which contains DNA in the form of about 6000 catenated circles. The kinetoplast DNA is 10% of the total cellular DNA and is the important site of action of some anti-trypanosome drugs such as ethidium. The kinetoplast is part of the single long mitochondrion which changes morphology during various stages of life cycle.

Most other organelles are those typical of any eucaryotic cell. At surface of the cell are sub-membranous pellicular microtubules which give the trypanosome its shape. These underlie a typical plasma membrane which is often covered by an electron-dense surface coat.

Trypanosomes are successful parasites which manage to escape the host's immune response; this happens by a very complex mechanism of antigen switching and it is the knowledge of this mechanism that has led to the first steps in developing an anti-trypanosome vaccine. They are polymorphic and may be long and slender, or short and stumpy. The stained flagellates measure 15 to 30 microns by 1.5 to 3.5 microns. The different forms of the parasite includes the following:



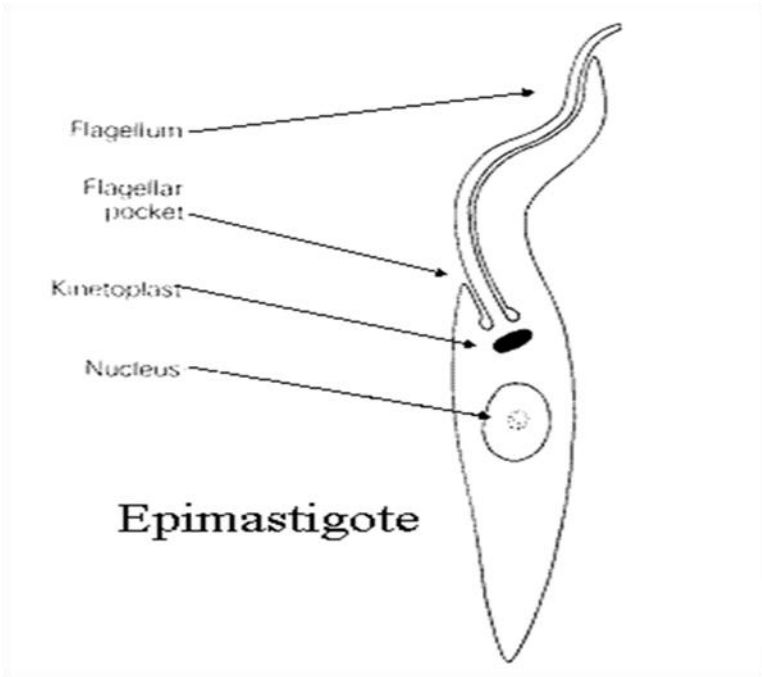
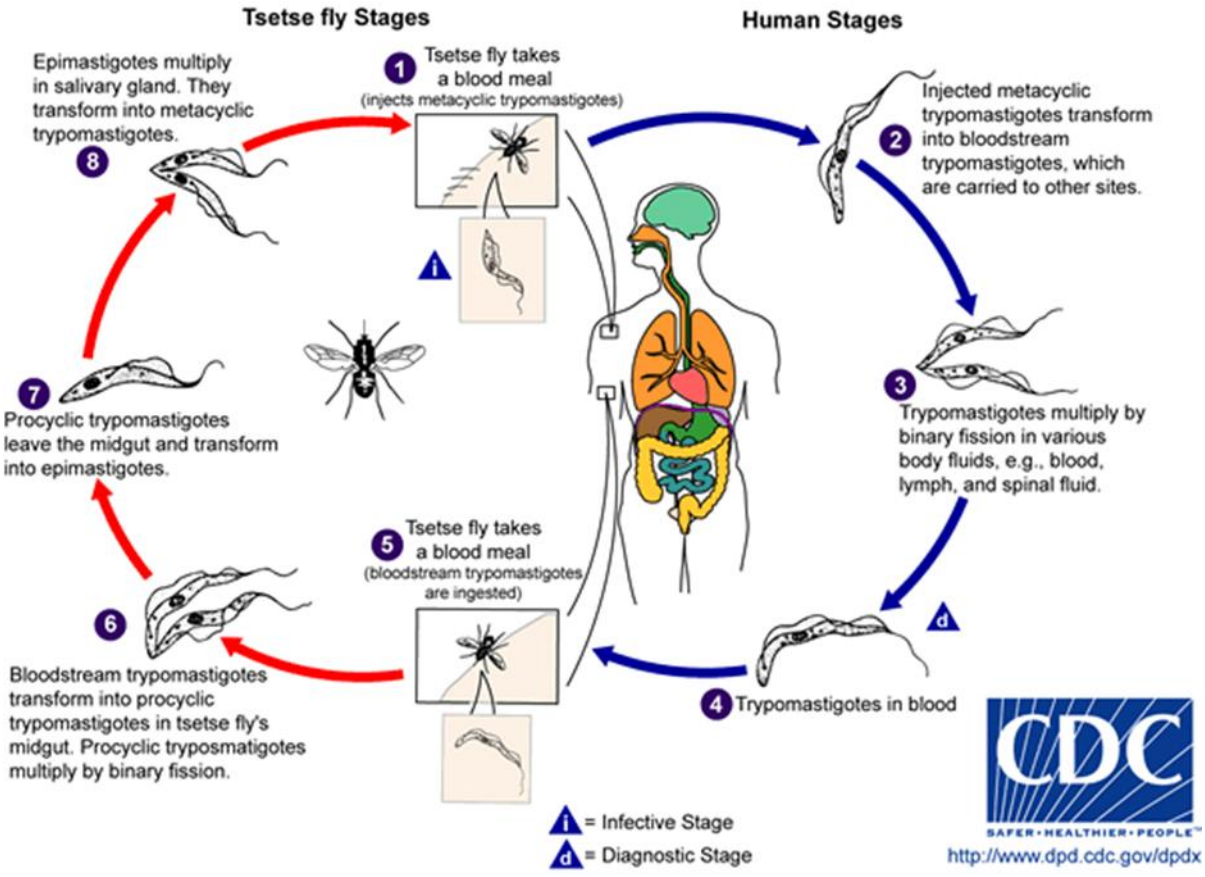


Figure 2.2: Stages of Trypanosomes

2.4.1 Life Cycle

The life cycle of trypanosome parasite (Trypanosoma brucei) is represented in the diagram below:



www.dpd.cdc.gov/dpdx

Figure 2.3: Life Cycle of African Trypanosome.

The entire life cycle of African trypanosomes is represented by extracellular stages. A tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission, leave the midgut, and transform into

epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission.

Live trypanosomes are easily recognized because of their very active wiggling movements, due to the ondulatory motion of the flagellum, which runs along the cell body and ends in front in a free tip.

The kinetoplast DNA is 10% of the total cellular DNA and is the important site of action of some anti-trypanosome drugs such as ethidium. The kinetoplast is part of the single long mitochondrion which changes morphology during various stages of life cycle.

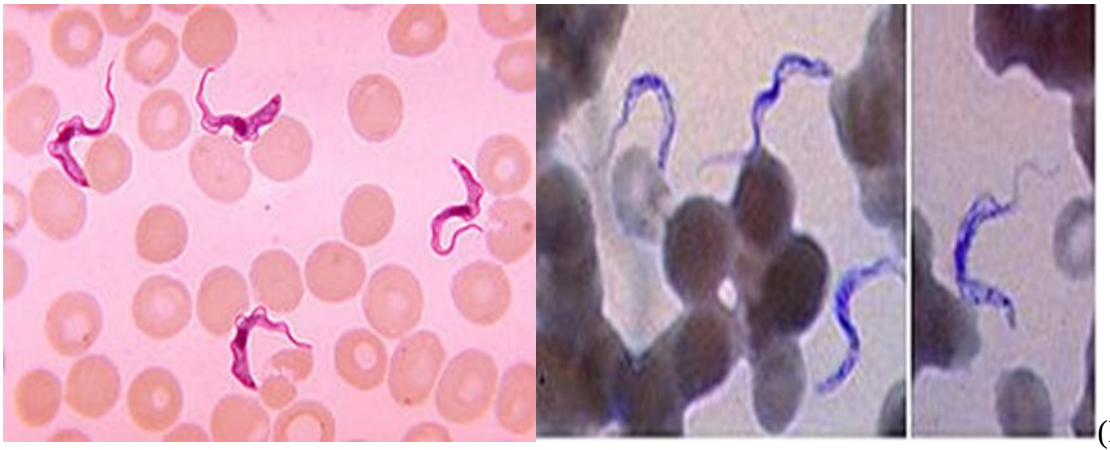


Plate 2.1: Blood Smear Showing Trypanosomes. (Hunt, 2004).

2.5 Development of Trypanosomiasis

While taking blood from a mammalian host, an infected tsetse fly injects metacyclic trypomastigotes into skin tissue. From the bite, parasites first enter the lymphatic system and then pass into the bloodstream. T. brucei have a specialized mechanism to overcome the obstacles of the mammalian immune system. Days after infection, host antibodies recognize surface glycoproteins that coat the protozoa—the

antigenic determinants of the organism—and kill the organisms by labeling them destruction. However a few protozoa escape destruction via a programmed system that changes their glycoprotein composition and thus enables them to evade immune recognition. Again, days later the immune system recognizes this change and mounts an immune response against the new glycoprotein. This cycle is repeated with a pattern of high parasite load followed by a period of low parasite load. For this reason, patients exhibit an irregular pattern of high-grade fevers followed by an afebrile period throughout the course of a systemic infection. For this reason, vaccine development has been thwarted. Inside the mammalian host, they transform into bloodstream trypomastigotes, and are carried to other sites throughout the body, reach other body fluids (e.g., lymph, spinal fluid), and continue to replicate by binary fission.

The most striking and obvious feature of trypanosome infection in susceptible hosts is anaemia. Scientists have been able to demonstrate that during infection of susceptible hosts, macrophages, which are usually thought of as playing a protective role in infections, act in a harmful manner by engulfing and destroying red and white blood cells and their progenitors in the bone marrow. The production of the cytokine IL1, which is produced by macrophages, increases in the early stages of the disease, suggesting that this cell system is activated during infection. It now appears that the activation may be partly responsible for the pathogenic effects of disease rather than protective responses to it. Haemodilution, osmotic and mechanical fragility of RBCs, extravascular haemolysis, decrease life span of RBCs and dyshaemopoiesis all have been stated by various research workers as pathogenic mechanisms in different animals (Clarkson, 1968; Jatkar and

Purohit, 1971; Anosa and Isoun, 1976; Dargie *et al*., 1979; Murray, 1979; Cox and Sale, 1983; Singh and Mishra, 1986).

There is no obvious reason why trypanosomes enter the brain of infected mammals, because here they cannot be taken up by tsetse flies during a blood meal. In terms of an evolutionary pressure, one may consider that parasites within the brain are hidden from the major parts of the immune system as a reservoir in case of an eradication of blood parasites. Tsetse flies are pool feeders, i.e. they cut the skin and suck up blood from the respective lesion. Interestingly, prostaglandin D2, which induces non-rem sleep if injected into the ventricle system (Hayaishi *et al*., 2004), is significantly increased in csf of late stage patients (Pentreath,1995). Trypanosomes are able to produce PGD2 (Kubata *et al*., 2007) ,that they are also use for cell density regulation (Duszenko *et al*., 2006). Bloodstream trypanomastigotes have been reported to possess the ability to traverse not just the blood-brain, but also the blood-cerebrospinal fluid (CSF) barriers (Wolburg *et al*., 2008). Choroid plexus epithelial cells secrete the cerebrospinal fluid that circulate in ventricular and subarachnoid spaces. Trypanosomes pass through the blood-cerebrospinal fluid barrier into the CSF and with the aid of IFN-γ-producing T cells invade choroid plexus early. They distrupt the choroid plexus epithelial barrier, IFN-γin CSF diffuses between ependymal cells into white matter to induce IP-10 and affect astrocytic basement membrane. The trypanosomes (and T cells) cross the BBB as a second wave (Kristensson, 2004).

Grab and his colleague observed a transient decay and recovery in transendothelial electric resistance during bloodstream-form parasite passage (Grab *et al*.,2004). They proposed that parasite binding at or near intercellular junctions of the BBB precedes

crossing via a paracellular route. In addition, we reported that T. b. gambiense induces oscillatory changes in the intracellular calcium ([Ca2+]i) of brain microvascular

endothelial cells (BMECs) and proposed that signaling events triggered by bloodstream-form parasites might render the endothelial cells permissive to T. brucei traversal (Grab et al.,2004). Based on previous findings that mechanical stimulation of a single cortical capillary endothelial cell can trigger calcium waves (Paemeleire et al., 1999), we initially proposed that actively swimming trypanosomes might be inducing changes in [Ca2+]i by mechanical stimulation of BMECs (Grab et al., 2004). We also suggested that bloodstream-form trypanosome proteases might subsequently process or degrade proteins at the intercellular junctions, enabling parasites to cross through the barrier between the cells (Grab *et al*.,2004). To date, the molecular players involved in parasiteinduced signaling and crossing of BBB are unknown (Olga *et al*.,2006).

In HAT infections, the tight junctions of the brain-endothelial barrier are not disrupted, and damage to the barrier is minimal, making it difficult to correlate CNS invasion with parasite-endothelium interactions (Dumas and Bouteille, 1997, Lonsdale-Eccles, and Grab, 2002). Although the process of CNS invasion is still poorly understood (Dumas and Bouteille, 1997; Lonsdale-Eccles, and Grab, 2002), a growing body of evidence from studies in animal models of the disease indicates that the parasites may cross the BBB directly. In experimental animals infected with African trypanosomes, the parasites appear early during infection in the choroid plexus and other circumventricular organs (Schultzberg, 1988) that lack a BBB. At later stages, the parasites penetrate the true BBB without apparent disruption of the endothelial tight junctions and enter the brain parenchyma. This was shown by double immunohistochemical labeling of parasites and

brain endothelial cells (Mulenga *et al*.,2001). Of further interest, Masocha *et al*. (2004) have shown that T. b. brucei cross the cerebral blood vessels of mice through interactions with endothelial cells that express laminin 8, suggesting that these parasites and leukocytes may traverse the intact BBB through the same or similar sites.

Human African trypanosomiasis, or sleeping sickness, is caused by the protozoan parasites Trypanosoma brucei brucei, and is a major cause of systemic and neurological disability throughout sub-Saharan Africa. Following early-stage disease, the trypanosomes cross the blood–brain barrier to invade the central nervous system leading to the encephalitic, or late stage, infection (Rodgers *et al*., 2009). Surprisingly, it has been shown that no trypanosomes observed within the parenchyma outside brain blood vessels and that brain infection depends on the formation of long slender trypanosomes. Cerebrospinal fluid as well as the stroma of the choroid plexus is a hostile environment for the survival of trypanosomes, which enter the pial space including the Virchow-Robin space via the subarachnoid space to escape degradation. As a result, trypanosomes do not intend to colonize the brain but reside near or within the glia limitans, from where they can re-populate blood vessels and disrupt the sleep wake cycles (Wolburg *et al*., 2012).

It has been demonstrated that kynurenine pathway catabolites are involved in the generation of the more severe inflammatory reaction associated with the late central nervous system stages of the disease and suggest that Ro-61-8048 or a similar drug may prove to be beneficial in preventing or ameliorating the post-treatment reactive encephalopathy (PTRE) when administered as an adjunct to conventional trypanocidal chemotherapy (Rodgers, *et al.,* 2009). Rodgers and his co-researchers were able to show a significant ($P = 0.0284$) reduction in the severity of the neuroinflammatory response

which was detected when the inhibitor was administered in animals exhibiting the more severe, late central nervous system stage, of the infection, using the mouse hippocampus and hypothalamus as a model.

2.6 Human African Trypanosomiasis

Human African trypanosomiasis takes two forms, depending on the parasite involved:

Trypanosoma brucei gambiense (T.b.g.) is found in west and central Africa. This form currently accounts for over 95% of reported cases of sleeping sickness and causes a chronic infection. A person can be infected for months or even years without major signs or symptoms of the disease. When symptoms emerge, the patient is often already in an advanced disease stage where the central nervous system is affected.

Trypanosoma brucei rhodesiense (T.b.r.) is found in eastern and southern Africa. Nowadays, this form represents under 5% of reported cases and causes an acute infection. First signs and symptoms are observed a few months or weeks after infection. The disease develops rapidly and invades the central nervous system.

Another form of trypanosomiasis occurs mainly in 21 Latin American countries. It is known as American trypanosomiasis or Chagas disease. The causal organism is a different species from those causing the African form of the disease.

In addition to the bite of the tsetse fly, the disease can be transmitted by:

- Mother-to-child infection: the trypanosome can sometimes cross the placenta and infect the fetus (Olowe,1975).

- Laboratories: accidental infections, for example, through the handling of blood of an infected person and organ transplantation, although this is uncommon.
- Blood transfusion .
- Sexual contact (This may be possible) (Rocha *et al.*,2004).

2.6.1 Diagnosis

A physical examination may show signs of inflammation of the brain and its covering, the meninges (meningoencephalitis). Tests includes:

- Blood smear
- Cerebrospinal fluid tests
- Complete blood count (CBC)
- Lymph node aspiration (Kirchoff, 2009).

2.6.2 Prevention

Two alternative strategies have been used in the attempts to reduce the African trypanosomiases (WHO, 2006):

- The primary method focuses on the eradication of the tsetse fly, which disrupts transmission rates by reducing the number of flies. Instances of sleeping sickness are being reduced by the use of the sterile insect technique.

- The second tactic is primarily medical or veterinary, and tries to reduce spread of the parasite by monitoring, prophylaxis, treatment, and surveillance to reduce the number of people or animals who carry the disease.

2.6.3 Treatments

- First line, first stage:

The current standard treatment for first-stage (haemolymphatic) disease includes:

Intravenous or intramuscular pentamidine (for T.b. gambiense), or intravenous suramin (for T.b. rhodesiense).

The drug eflornithine — previously used only as an alternative treatment for sleeping sickness due to its labour-intensive administration — was found to be safe and effective as a first-line treatment for the disease in 2008, according to the Science and Development Network's sub-Saharan Africa news update (Robinson *et al*.,2009). Researchers tracked over 1,000 adults and children at a centre in Ibba, Southern Sudan—the first use of eflornithine on a large scale— and it was highly effective in treating the issue.

According to a treatment study of Trypanosoma gambiense-caused human African trypanosomiasis, use of eflornithine resulted in fewer adverse events than treatment with melarsoprol (Chappuis *et al.*,2005).

- First line, second stage:

  The current standard treatment for second-stage (neurological phase) disease is:

  Intravenous melarsoprol 2.2 mg/kg daily for 12 consecutive days (Burri *et al.*,2000).

- Alternative first line therapies include:

  Intravenous melarsoprol 0.6 mg/kg on day 1, 1.2 mg/kg IV melarsoprol on day 2, and 1.2 mg/kg/day IV melarsoprol combined with oral 7.5 mg/kg nifurtimox twice a day on days 3 to 10 (Bisser *et al*.,2007)or

  Intravenous eflornithine 50 mg/kg every six hours for 14 days (van Nieuwenhove et al.,2005).

Combination therapy with eflornithine and nifurtimox is safer and easier than treatment with eflornithine alone, and appears to be equally or more effective. It has been recommended as first-line treatment for second-stage T. b. gambiensis disease (Priotto *et al.*,2009).

Current treatments are old and toxic or very difficult to use. Unfortunately, the very trusted melarsoprol, an arsenic derivative, is highly toxic and treatment can result in the development of an extremely severe post-treatment reactive encephalopathy (PTRE) in about 10% of patients with a 50% mortality rate (Pepin and Milord, 1991, 1994). Elornithine has to be given by 56 infusions in a hospital, one every 2hours for 14days, which is virtually impossible in many of the worst-affected areas (Sarah, 2012).

“Millions of lives in Africa are at risk from this disease; it is important we continue to invest in reseach that will unearth treatments that are affordable, effective and that will save millions of lives” (WHO,2012).

2.7 Animal trypanosomiasis

Other parasite species and sub-species of the Trypanosoma genus are pathogenic to animals and cause animal trypanosomiasis in wild and domestic animal species. In cattle the disease is called Nagana, a Zulu word meaning "to be depressed" (WHO, 2012).

Animals can host the human pathogen parasites, especially T.b. rhodesiense; thus domestic and wild animals are an important parasite reservoir. Animals can also be infected with T.b. gambiense and act as a reservoir. However the precise epidemiological role of this reservoir is not yet well known. The disease in domestic animals, particularly cattle, is a major obstacle to the economic development of affected rural areas (WHO, 2012). African Animal Trypanosomiasis (AAT) affects domestic livestock in sub-Saharan Africa and consequently has a severe economic impact (Barrett *et al*., 2007). AAT is widely spread affecting 40 countries situated in regions that could potentially be the most productive (Kappmeier *et al*., 1998). The main pathological symptoms of animal trypanosomosis are weight loss, anaemia, and immunosuppression, but the mechanisms involved are poorly understood. It is estimated that 50 million cattle and 70 million small ruminants are at risk, costing the continent between 1.5 and 5 billion US dollars per annum. In terms of severity and consequences for productivity, T. congolense is the main causative agent of AAT (Van den Bossche, 2001).

2.8 Trypanosoma brucei brucei

T. brucei brucei belong to the order KINETOPLASTIDA, so-called because of the large DNA-containing structure, the kinetoplast, found at the base of the flagellum.It transmit the acute form of nagana to cattle, antelopes, camels and horses, through subspecies of glossina which includes:

G. palpalis

G. morsitans

G. austeni

G. swynnertoni

G. pallidipes

G. longipalpis

G. tachinoides

G. brevipalpis

(Hunt, 2004).

2.9 Effect of Trypanosomiasis

The first clinical manifestation of African trypanosomiasis occurs a few days after infection as a chancre at the site of tsetse fly inoculation. This is due to the localized proliferation of the pathogens within the subcutaneous tissue. Incubation period for T.b. rhodesiense may be two to three weeks, while the incubation period for the Gambian

species may last several weeks to months. With the conclusion of the incubation period, the organisms have already disseminated into the bloodstream, leading to the emergence of a characteristic intermittent fever pattern that correlates directly with high versus low levels of parasitemia. The reason for the oscillating levels of parasite load is linked to the ability of the organisms to change their variable surface glycoproteins (VSGs) and evade the host's immune system. Lymphadenopathy, the swelling of lymph nodes, especially in the posterior cervical nodes (on the back of the neck) is characteristic sign of African sleeping sickness and is termed Winterbottom's sign. Invasion of the central nervous system (CNS) occurs within several weeks in the Rhodesian species and months to even years in the Gambian species of trypanosomiasis.

In classical human sleeping sickness, the parasites invade the CNS, and the infected individuals suffer white matter encephalitis with concomitant psychiatric and motor disorders. The neurological manifestations of HAT appear after the invasion of the CNS by the parasite (Kennedy, 2004).

"From the beginning of Arab and European influence in the hinterland of tropical Africa, trypanosomiasis of man and animals has curbed the realization of human ambitions and the mobilization of the continent's vast resources" (Gasser,1963). The speed to which the symptom of T. brucei expresses itself, especially the neurological phase, is very alarming. Though, it is said that the appearance of T. brucei in the CNS could cause many neurological disoders within several weeks in the Rhodesian species and months to even years in the Gambian species of trypanosomiasis. Symptoms include headache, stiff neck, sleep disturbance, and depression, followed by progressive mental deterioration, focal seizures, tremors, and palsies. This progresses to coma and the ultimate death of

the patient often secondary to pneumonia or sepsis. Without treatment, African trypanosomiasis is a universally fatal illness (Chevries *et al*.,2005). How trypanosoma brucei manage to cause all these neurological damage is still elusive.

Acute increase in parasitemia accounts for the decrease in AChE activity in brainand blood which implies that ACh will be high in the motor division and autonomic ganglia. The excess Ach accumulates therefore making the neurons to constantly fire, causing the muscles in the animals to develop paralysis and asphyxiation which is a common symptom observed at the latter stage of the disease (Habila *et al*., 2012).

The PTRE experienced when the parasite crosses the blood-brain barrier is characterized neuropathologically by the development of a meningoencephalitis with diffuse astrocytosis and the presence of macrophages, lymphocytes and plasma cells in cerebral white matter as well as perivascular cuffing (Adams *et al*., 1986; Kennedy, 2006).

The activation of kynurenine pathway by *trypanosoma brucei brucei* infection, with the generation of neuroactive products, raises the possibility that it could play a role in the development of the neuroinflammatory response associated with trypanosome infection and drug treatment. The study arose out of the interest in identifying novel pathways that might be important in CNS HAT, and their potential for identifying possible inhibitors to ameliorate CNS disease (Rodgers *et al*., 2009).

The severity of the neuroinflammatory reaction in trypanosomiasis could be neuropatholgically graded using a neuropathological grading scale implemented in previous studies (Kennedy *et al*., 1997). Briefly, slides were examined by two independent assessors in a blinded fashion. The severity of the reaction was graded on a

scale of 0 to 4 where 0 represents a normal brain with no sign of pathological change, Grade 1 shows a mild meningitis, Grade 2 demonstrates a moderate meningitis with some degree of perivascular cuffing, Grade 3 shows a more severe meningitis with cuffing and some infiltration of the neuropil by inflammatory cells and Grade 4 is characterized by a severe meningoencephalitis with the presence of inflammatory cells in the brain parenchyma.

Neurological features of late-stage HAT include the characteristic sleep disorder with alteration of sleep structure, a variety of neuropsychiatric symptoms, both motor and sensory disturbances, and progressive cerebral oedema, incontinence and coma leading to death (Kennedy, 2008).

Gabriella demonstrated that *T. b. brucei* dysregulates the endogenous rhythm in SCN activity, which probably alters the circadian output and may be manifested as a fragmentation of the sleep-wake cycle. Further, the SCN contain glutamatergic synapses that display an increase in activity during the subjective day *in vitro.* This activity is decreased in slices from trypanosome-infected rats, possibly explainingbthe observed alteration in spontaneous firing. Cytokines released during trypanosome-infections, such as IFN-γ, may affect protein expression of glutamate receptors and glutamatergic postsynaptic transmission via its receptor, which is located in the ventrolateral region of the SCN (Lundkvist, 2001).

Neuroscientist in the university of Verona were able to observe sleep and rhythm changes at the time of *Trypanosoma brucei* invasion of the brain parenchyma in the rat. They showed that sleep pattern and sleep-wake organization plus rest-activity and body

temperature rhythms exhibited early quantitative and qualitative alterations, which became marked around the time interval crucial for parasite neuroinvasion or shortly after. Data derived from actigrams showed close correspondence with those from hypnograms, suggesting that rest-activity could be useful to monitor sleep-wake alterations in African trypanosomiasis (Seke-Etet *et al*., 2012).

2.10 Calbindin D28k

Calbindin D28k is a high-affinity calcium binding protein found to be widely distributed in the nervous system where it has been shown to modulate calcium ion signaling, even though it specific mode of function is largely unclear. Calbindin D28K, also known as calbindin, CALB1, D-28K or vitamin D-dependent calcium-binding protein, is a protein with 6 EF-hand domains, 4 of which are active calcium-binding domains. Calbindin belongs to a family of calcium-binding proteins which also includes calmodulin, parvalbumin, troponin C and S100 (Christakos *et al.*, 1989; Gross and Kumar, 1990). It contains 4 active calcium-binding domains, and 2 modified domains that have lost their calcium-binding capacity. They were originally described as vitamin D-dependent calcium-binding proteins in the intestine and kidney in the chick and mammals. They are now classified in different sub-families as they differ in the number of Ca2+ binding EF-hand sites. Calbindin D28k is a high molecular mass form of 28,000 Daltons which acts as calcium buffer and calcium sensor and can hold four calcium ions in the EF-hands of loops EF1, EF3, EF4 and EF5 (Kojetin *et al*., 2006). It is a vitamin D responsive gene in many tissues, in particular the chick intestine, where it has a clear

function in mediating calcium absorption(Wasserman and Fullmer, 1999). The sequence of Calbindin is 263 residues in length and has only one chain. The sequence consists mostly of alpha helicies but beta sheets are not absent. According to the PDB it is 44% helical with 14 helices containing 117 residues, and 4% beta sheet with 9 strands containing 13 residues.

It is expressed in brain, ovary, uterus, testis, pancreas, liver, kidney and intestine, it acts as a calcium-buffering agent and alters the activity of the plasma membrane ATPase. In neuronal cells, Calbindin D28K modulates calcium channel activity, calcium transients and intrinsic neuronal firing activity. It is also expressed in a number of neurocrine cells, particularly in the cerebellum and it is encoded in humans by the CALB1 gene. More than ten calcium-binding proteins have been found in the nervous system (Baimbridge *et al*., 1992). It is expressed in distinct cell types throughout the central nervous system and in nonneuronal tissue (Baimbridge, 2002). The localization of calbindin is within the cell body, dendrites, axons of specific populations of nerve cells and the nucleus (German *et al*., 1997).

In the brain, its synthesis is independent of vitamin-D. In neuronal cells, calbindin D28k modulates calcium channel activity, calcium trancients and intrinsic neuronal firing activity. Also, it has been implicated to play a key role in apoptosis and micro tubule function (Hitomi *et al*., 1996). The exact functional role of calbindin-D28k has not yet been established (Christakos *et al*., 1997). It has been described as a carrier protein, which facilitates the trans-cellular Ca+2 transport (Bronner and Stein, 1988; Christakos *et al*., 1997) and as a buffer protein, which prevents intracellular Ca+2 concentrations from reaching toxic levels during Ca+2 transport (Roth *et al*., 1981; Bronner and Stein, 1988;

Johnson and Kumar, 1994) and thus protect nerve cells (Heizman and Braun, 1992; Wasserman and Fullmer, 1983). Light and electron microscopy have clearly demonstrated the existence of calbindin D28k in presynaptic nerve terminals, where it exists in up to millimolar concentrations. It is therefore tempting to speculate that calbindin D28k also plays a role in modulating synaptic physiology(Cheng *et al.*,1994).

A considerable attention has been focused on the localization of calcium-binding proteins (CBP) because they serve as good markers to distinguish subpopulations of neuron (Celio, 1990) and because their distribution is altered in many neurodegerative disorders (Reynolds *et al*., 2001). Calcium-binding proteins have also been used to identify separate population of GABAergic neurons in many areas of the cerebral cortex (Gonchar *et al*., 1997).

Calbindin D28k have been located in the visual cortex of several mammals including humans (Letinic, 1998), rats and mouse (Gonchar,1997) and the lack of calbindin-D28k alters response of the murine circadian clock to light (Stadler *et al*., 2010). A strong stimulus adjusting the circadian clock to the prevailing light-dark cycle is light. However, the circadian clock is reset by light only at specific times of the day. The mechanism mediating such gating of light input to the CNS are not well understood. There is evidence that calcium ions play an important role in the light-mediated resetting of the circadian clock. The absence of calbindin-D28k influences calcium ion signaling, and hence might alter transmissionnof light information to the circadian clock in neurons of the suprachiasmatic nuclei (SCN) (Stadler, *et al*.,2010).

The mammalian circadian clock influences a multitude of physiological processes such as cardiovascular activity, sleep/wakefulness, metabolism, and brain function (Roenneberg *et al*.,2003). Correct synchronization of the circadian clock is mediated by light. Light information is transmitted by the retina via the retenohypothalamic tract (RHT) to the suprachiasmatic nuclei (SCN) harboring the main clock in the brain and thereby the SCN clock is synchronized to environmental time.(Dardente *et al.*,2007). Circadian rhythm is any biological process that displays an endogenous, entrainable oscillation of about 24hours. These rhythms are driven by a circadian clock. The primary circadian "clock" is located in the suprachiasmatic nucleus (or nuclei) (SCN), a pair of distinct groups of cells located in the hypothalamus.

2.12 The hippocampal formation

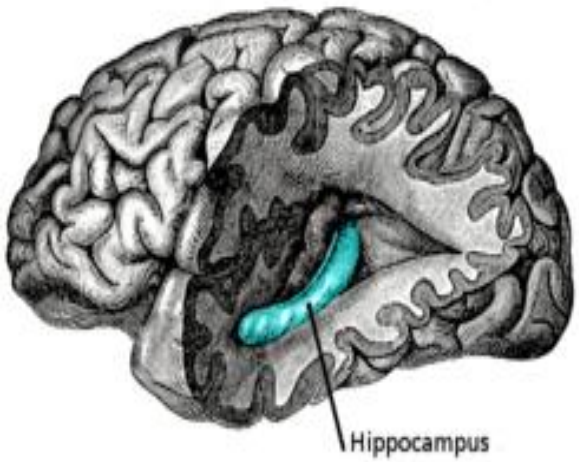
The hippocampal formation is collectively made up of hippocampus, dentate gyrus and the subiculum, which makes up part of the limbic system (limbic region). The hippocampus and the dentate gyrus belong to the allocortex and have a simplified laminar pattern compared with the neocortex (Brodal,1992). The subiculum showed a graded variation in structure from a four, through a five, to a modified six layered type of cortex, where it merges with the surrounding neocortex (Warwick and Williams, 1989).

2.12.1 Hippocampus

The hippocampus is a major component of the brain in humans and other vertebrates which belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. Humans and other mammals have two hippocampi, one in each side of the brain. Some researchers view 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) (Square, 1992). The hippocampus is a part of the cerebral cortex, and in primates is located in the medial temporal lobe, underneath the cortical surface. It contains two main interlocking parts: Ammon's horn and the dentate gyrus (Pearce,2001). Since defferent neuronal cell types are neatly organised into layers in the hippocampus, it has frequently been used as a model system for studying neuroscience.

The hippocampus is the part of the brain that is involved in memory forming, organizing, and storing. It is a limbic system structure that is particularly important in forming new memories and connecting emotions and senses, such as smell and sound, to memories.

Topologically, the surface of a cerebral hemisphere can be regarded as a sphere with an indentation where it attaches to the midbrain. The structures that line the edge of the hole collectively make up the so-called limbic system (Latin limbus = border), with the hippocampus lining the posterior edge of this hole.



(Nolte, 2002)

Figure 2.4: The Hippocampus

The hippocampus is a horseshoe shaped paired structure, with one hippocampus located in the left brain hemisphere and the other in the right hemisphere. Anatomically, the hippocampus is an elaboration of the edge of the cerebral cortex. It is connected to parts of the brain that are involve with emotional behaviours : The septum, the hypothalamic mammillary body, and the anterior nuclear complex in the thalamus ; so its role as a limbic organ can not be completely dismissed (Amaral et al.2006). Directionally, the hippocampus is located within the temporal lobes, adjacent to the amygdala. The hippocampus acts as a memory indexer by sending memories out to the appropriate part of the cerebral hemisphere for long-term storage and retrieving them when necessary (Nolte, 2002).

2.12.3 Hippocampal Histology

The hippocampus as a whole has the shape of a curved tube, which has been analogized variously to a seahorse, a ram's horn (Cornu Ammonis, hence the subdivisions CA1 through CA4), or a banana (Amaral and Lavenex, 2006).

Anatomically, the hippocampus is superior to the subiculum and medial part of the parahippocampal gyrus, where it forms a curved elevation of about 5cm long, extending throughout the entire length of the floor of the inferior horn of the lateral ventricle. Its anterior extremity is expanded, and at this point its margin sometimes presents two or three shallow grooves with intervening elevations, giving a paw-like appearance called “pes hippocampi” (Amaral and Insuasti, 1990). The ventricular surface is convex in coronal section, covered by ependymal. Beneath the ventricular surface, white fibres called alveus converge medially upon a longitudinal projecting bundle of white fibre called “the fimbria of the fornix” passing medially from the line of the collateral sulcus; the parahippocampal gyrus merges with the subiculum. The subiculum curves superomedially to reach the inferior surface of the dentate gyrus (Amaral and Insausti, 1990).

The entorhinal cortex (EC), the greatest source of hippocampal input and target of hippocampal output, is strongly and reciprocally connected with many other parts of the cerebral cortex, and thereby serves as the main "interface" between the hippocampus and other parts of the brain.

The superficial layers of the EC provide the most prominent input to the hippocampus, and the deep layers of the EC receive the most prominent output.

Within the hippocampus, the flow of information is largely unidirectional, with signals propagating through a series of tightly packed cell layers, first to the dentate gyrus, then to the CA3 layer, then to the CA1 layer, then to the subiculum, then out of the hippocampus to the EC. Each of these layers also contains complex intrinsic circuitry and extensive longitudinal connections.

Starting at the dentate gyrus and working inward along the S-curve of the hippocampus means traversing a series of narrow zones. The first of these, the dentate gyrus (DG), is actually a separate structure, a tightly packed layer of small granule cells wrapped around the end of the hippocampus proper, forming a pointed wedge in some cross-sections, a semicircle in others. Next come a series of Cornu Ammonis 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. After this comes 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 hippocampus proper plus dentate gyrus and subiculum (Amaral and Lavenex, 2006).

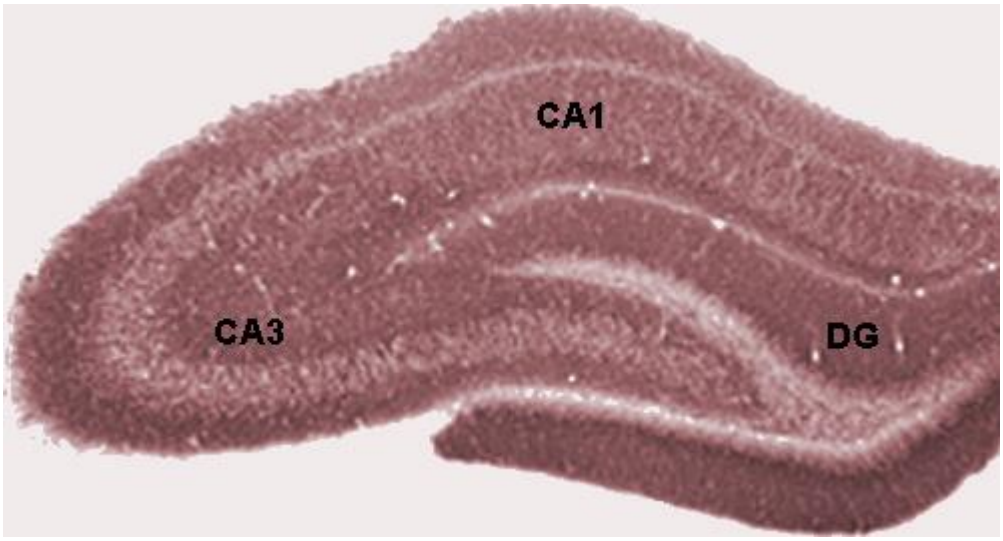


Plate 2.2: The Hippocampus. (Amaral and Lavenex, 2006).

The cortical region adjacent to the hippocampus is known collectively as the parahippocampal gyrus (or parahippocampus).

Cut in cross section, the hippocampus is a C-shaped structure that resembles a ram's horns. The name cornu ammonis refers to the Egyptian deity Amun, who has the head of a ram. The horned appearance of the hippocampus is caused by cell density differentials and the existence of varying degrees of neuronal fibers.

In rodents, the hippocampus is positioned so that, roughly, one end is near the top of the head (the dorsal or septal end) and one end near the bottom of the head (the ventral or temporal end), as seen in the figure below:

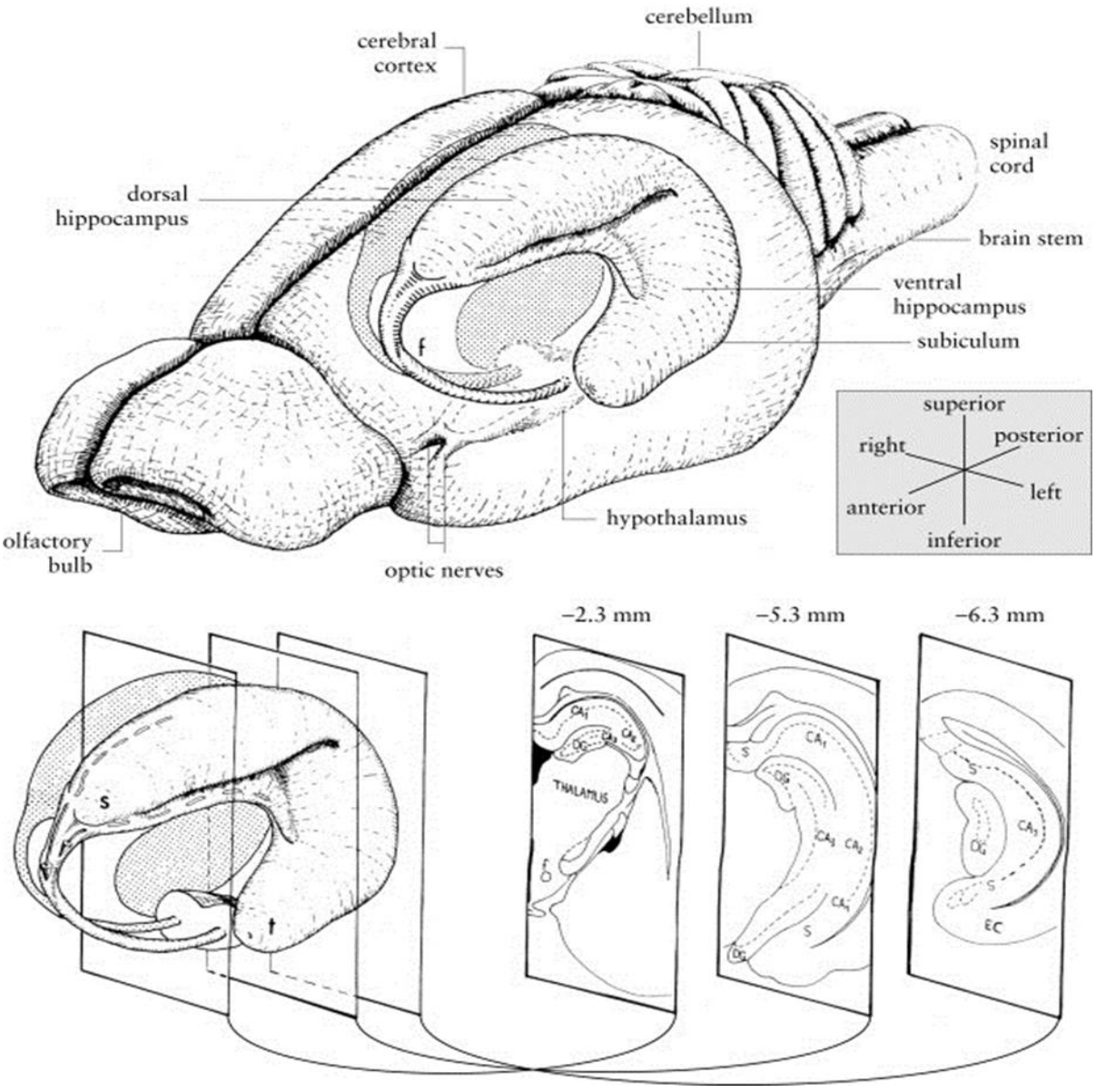


Figure 2.5: The Positions of Hippocampus. (Amaral and Lavenex, 2006).

As shown in the figure, the structure itself is curved and subfields or regions are defined along the curve, from CA4 through CA1 (only CA3 and CA1 are labeled).

Intesive histological studies of the cornu Ammonis (CA) reveals that it regions are structured depthwise in clearly defined strata (or layers):

- The alveus is the deepest layer and contains the axons from pyramidal neurons, passing on toward the fimbria/fornix, one of the major outputs of the hippocampus.
- Stratum oriens (str. oriens) is the next layer superficial to the alveus. The cell bodies of inhibitory basket cells and horizontal trilaminar cells, named for their axons innervating three layers-- the oriens, pyramidal, and radiatum are located in this stratum. The basal dendrites of pyramidal neurons are also found here, where they receive input from other pyramidal cells, septal fibers and commissural fibers from the contralateral hippocampus (usually recurrent connections, especially in CA3 and CA2.) In rodents the two hippocampi are highly connected, but in primates this commissural connection is much sparser (Anderson, 2007).
- Stratum pyramidale (str. pyr.) contains the cell bodies of the pyramidal neurons, which are the principal excitatory neurons of the hippocampus. This stratum tends to be one of the more visible strata to the naked eye. In region CA3, this stratum contains synapses from the mossy fibers that course through stratum lucidum. This stratum also contains the cell bodies of many interneurons, including axo-axonic cells, bistratified cells, and radial trilaminar cells.
- Stratum lucidum (str. luc.) is one of the thinnest strata in the hippocampus and only found in the CA3 region. Mossy fibers from the dentate gyrus granule cells course through this stratum in CA3, though synapses from these fibers can be found in str. pyr.

- Stratum radiatum (str. rad.), like str. oriens, contains septal and commissural fibers. It also contains Schaffer collateral fibers, which are the projection forward from CA3 to CA1. Some interneurons that can be found in more superficial layers can also be found here, including basket cells, bistratified cells, and radial trilaminar cells.
- Stratum lacunosum (str. lac.) is a thin stratum that too contains Schaffer collateral fibers, but it also contains perforant path fibers from the superficial layers of entorhinal cortex. Due to its small size, it is often grouped together with stratum moleculare into a single stratum called stratum lacunosum-moleculare (str. l-m.) (Anderson, 2007).
- Stratum moleculare (str. mol.) is the most superficial stratum in the hippocampus. Here the perforant path fibers form synapses onto the distal, apical dendrites of pyramidal cells (Anderson, 2007).
- The hippocampal sulcus (sulc.) or fissure is a cell-free region that separates the CA1 field from the dentate gyrus. Because the phase of recorded theta rhythm varies systematically through the strata, the fissure is often used as a fixed reference point for recording EEG as it is easily identifiable (Anderson, 2007).

2.12.6 Functions of the hippocampus

The functional role of the hippocampal formation is dependent on its connections with various cortical association areas and with the limbic structures such as the cingulate

gyrus and the septal nuclei and with the hypothalamus (Brodal, 1992). Its functions includes

Consolidation of New Memories

Emotional Responses

Navigation

Spatial Orientation

2.12.7 Role in memory

Psychologists and neuroscientists generally agree that the hippocampus has an important role in the formation of new memories about experienced events (episodic or autobiographical memory) (Squire and Schacter, 2002).

Some researchers view 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 1992).

Damage to the hippocampus does not affect some types of memory, such as the ability to learn new motor or cognitive skills (playing a musical instrument, or solving certain types of puzzles, for example). This fact suggests that such abilities depend on different types of memory (procedural memory) and different brain regions. Furthermore, amnesic patients frequently show "implicit" memory for experiences even in the absence of

conscious knowledge. For example, a patient asked to guess which of two faces they have seen most recently may give the correct answer the majority of the time, in spite of stating that they have never seen either of the faces before. Some researchers distinguish between conscious "recollection", which depends on the hippocampus, and "familiarity", which depends on portions of the medial temporal cortex (Diana *et al*., 2007).

2.12.8 Role in spatial memory and navigation

Studies conducted on freely moving rats and mice have shown that many hippocampal neurons have "place fields", that is, they fire bursts of action potentials when a rat passes through a particular part of the environment. Evidence for place cells in primates is limited, perhaps in part because it is difficult to record brain activity from freely moving monkeys. Place-related hippocampal neural activity has been reported in monkeys moving around inside a room while seated in a restraint chair (Matsumura *et al.*, 1999); on the other hand, Edmund Rolls and his colleagues instead described hippocampal cells that fire in relation to the place a monkey is looking at, rather than the place its body is located (Rolls and Xiang, 2006).

In humans, place cells have been reported in a study of patients with drug-resistant epilepsy who were undergoing an invasive procedure to localize the source of their seizures, with a view to surgical resection. The patients had diagnostic electrodes implanted in their hippocampus and then used a computer to move around in a virtual reality town (Ekstrom *et al.*, 2003).

Place responses in rats and mice have been studied in hundreds of experiments over four decades, yielding a large quantity of information (Moser *et al*., 2008).

The discovery of place cells in the 1970s led to a theory that the hippocampus might act as a cognitive map—a neural representation of the layout of the environment (O'Keefe, and Nadel, 1978). Several lines of evidence support the hypothesis. It is a frequent observation that without a fully functional hippocampus, humans may not remember where they have been and how to get where they are going: getting lost is one of the most common symptoms of amnesia (Chiu, 2004). Studies with animals have shown that an intact hippocampus is required for some spatial memory tasks, particularly ones that require finding the way to a hidden goal (Clark *et al.,* 2005). The "cognitive map hypothesis" has been further advanced by recent discoveries of head direction cells, grid cells, and border cells in several parts of the rodent brain that are strongly connected to the hippocampus (Solstad *et al.,* 2008).

Brain imaging shows that people have more active hippocampi when correctly navigating, as tested in a computer-simulated "virtual" navigation task (Maguire *et al.*, 1998). Also, there is evidence that the hippocampus plays a role in finding shortcuts and new routes between familiar places. For example, London's taxi drivers must learn a large number of places and the most direct routes between them (they have to pass a strict test, The Knowledge, before being licensed to drive the famous black cabs).

A study at University College London by (Maguire *et al.,* 2000) showed that part of the hippocampus is larger in taxi drivers than in the general public, and that more experienced drivers have bigger hippocampi. Whether having a bigger hippocampus

helps an individual to become a cab driver, or if finding shortcuts for a living makes an individual's hippocampus grow is yet to be elucidated. Maguire *et al* examined the correlation between size of the grey matter and length of time that had been spent as a taxi driver, and found a positive correlation between the length of time an individual had spent as a taxi driver and the volume of the right hippocampus. It was found that the total volume of the hippocampus remained constant, from the control group vs. taxi drivers. That is to say that the posterior portion of a taxi driver's hippocampus is indeed increased, but at the expense of the anterior portion.

There have been no known detrimental effects reported from this disparity in hippocampal proportions (Maguire *et al*., 2000). It can be distinguished as a zone where the cortex narrows into a single layer of very densely packed neurons, which curls into a tight S shape.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Materials

3.1.1 Experimental Animals

Thirty (30) adult Wistar rats (male) were procured from the Pharmacology Animal House, Faculty of Pharmaceutical sciences, Ahmadu Bello University, Zaria, Kaduna, Nigeria.

The Wistar rats were transferred and housed in wired cages in the Animal House of the Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria, and allowed to acclimatize for four weeks prior to the start of the experiments.

All animals were given pelletized feed (Grand Cereals and Oil Mills Limited, Plateau State, Nigeria) and water *ad libitum*. Rats were weighed at the beginning, during and at the end of the study.

3.1.2 Equipment

- **Morris Water Maze Tank and Platform**

A local Morris water maze(MWM) tank of 1.2 meter in diameter and 60 centimeters deep and a platform was constructed in Sabon Gari market, Zaria, Nigeria.

- Other materials used in the course of the study include: digital weighing balance, cages, water bottles, saw dust for bedding, microscope, glass slides, cover slips,

stains, fixative and bottles for fixing tissues, dissecting set, beakers and syringes, digital venirecalliper, EDTA bottles,Capillary tubes, oven, and microtome.

3.1.3 Reagents

Reagents used includes primary antibody (anti-calbindin),eosin, haematoxylin, mercuric oxide, potassium alum, graded absolute alcohol, 95% alcohol, 70% alcohol, Xylene, paraffin wax, Anti-calbindin antibody, 10% formal saline, Bouin's fluid, and Stains.

3.1.4 Parasites

Trypanosoma brucei brucei (Kilifi strain) were gotten from the Protozology laboratory, Department of Veterinary Parasitology and Entomology,Faculty of Veterinary Medicine, A. B. U Zaria.

3.2 Methodology

3.2.1 Screening of Animals

All the rats were sccreened for trypanosomal infection using the wet blood smear preparation. Blood samples were taken from a nip at the tail-tip of the animals and transferred to a glass slide. A thin blood smear was made and covered with a cover slip before it was examined under a light microscope. This was done twice:

- Immediately after procurement and
- Just before the experinmental infection with trypanosome brucei brucei, to ensure that the animals were not infected already.

3.2.2 Experimental Designed

The 30 adult male Wistar rats were divided based on their weights into two groups. Group 1(n=6): uninfected (Control) and Group 2(n=24): infected. Group 2 was further

divided into four sub-groups: a, b, c, and d based on the time course of infection: Six animals were sacrificed at 3, 7, 14, and 21 days post infection.

Blood parameters of the animals were examined using the automated haematological analyzer.

The animals were then subjected through the Morris water maze training method and the latency time it took them to locate the hidden platform were obtained.

Table 3.2: Group 1(Control)

Rats	Weights(g)
1	392
2	181
3	180
4	168
5	155
6	112

N=6 rats

Table 3.3: Group 2a (3days post-infection)

Rats	Weights(g)
1	328
2	205
3	188
4	179
5	177
6	113

N=6rats

Table 3.4: Group 2b (7days post-infection)

Rats	Weights(g)
1	289
2	207
3	191
4	183
5	168
6	152

n=6 rats

Table 3.5: Group 2c (14days post-infection)

Rats	Weights(g)
1	228
2	220
3	205
4	189
5	185
6	161

n=6 rats

Table 3.6: Group 2d (21days post-infection)

Rats	Weights(g)
1	223
2	221
3	193
4	191
5	188
6	174

n=6 rats

3.2.5 Morris Water Maze Test

Morris water maze (MWM) test was used to test the spartial learning and memory of rats, according to he established protocol (Morris, 1981).

All animals were subjected to MWM training for 14 days, on how to escape danger using a platform and the latency time to find the hidden platform was recorded.

The MWM constituted of a circular tank (diameter: 1.2m; height: 60cm) filled with to a depth of 30cm and a 10cm diameter platform. The escape platform was made to be 1cm higher than the level of water at the beginning of the training. As the animals finds it easy to locate the platform, more water was added to about 0.5cm above the platform to make it more hidden. The animals were placed gently into the pool of water: back-end, first to avoid stress, and facing the pool side to avoid bias. The time taken to reach the platform (latency) was recorded using a stop watch. Habituation to the pool was done and rats were trained in seven(7) trials for visual cue discrimination.

3.2.6 Re-weighing of animals

All animals were re-weighed using a digital weighing balance; this is done in order to ascertain the exact weight of animals just before infection with parasites.

3.2.7 Infection of Animals

An innoculum dose of 1×10^6 trypanosomes per 1ml volume prepared in normal saline was used to infect the rats in group 2 via intraperitoneal(IP) route, while the group 1 rats were given 1ml of normal saline intraperitoneally(Ohaeri, 2010). *T. brucei brucei*

infected blood was collected from highly infected rat into a tube containing EDTA. The innoculum dose was calculated using Herbert and Lumsden rapid matching method of 1976; the calculation for the inoculum dose is indicated in **appendix II**.

3.2.8 Parasitemia Estimation

The presence and degree of parasitemia was determined 24hrs and 3days post-infection and then daily from the fourth day onwards for each rat. This was done by the rapid matching technique method(Herbert and Lumsden, 1976). A drop of blood was collected from the tail and placed on a glass slide, then a thin blood smear was prepared and examined under a light microscope with a ×400 magnification.

3.2.9 Weighing of animals

All animals were weighed using a digital weighing balance; this is done in order to determine the exact weight of animals just before sacrifice.

3.2.10 Morris Water Maze Test

The animal's ability to remember how to escape danger were tested, this was shown by their ability to locate the hidden platform (latency period). This was done after 3days for sub-group 2A, after 7days for sub-group 2B, 14days for sub-group 2C and 21days for group 1 and sub-group 2C.

3.2.11 Hematological Analysis

- Pre-Infection Blood Analysis

Blood samples were taken from the infra-orbital vein of each animal for hematological analysis. This was done by the infra-orbital puncture technique, using a fine capillary tube, blood samples were collected into EDTA bottles and transfered to automated hematological analyzer(Syntex Rx-21N Autoanalyser) for the analysis and the data obtained were recorded.

- Post-Infection Blood Analysis

Animals were anaesthesized with chloroform and sacrificed immediately, with the heart still pumping, blood was collected via cardiac puncture. The blood samples were transferred into EDTA bottles and analysed in an automated hematological analyzer (Syntex Rx-21N Autoanalyser) for blood cell count and other analysis. This was done after 3days for sub-group 2A, after 7days for sub-group 2B, 14days for sub-group 2C and 21days for group 1 and sub-group 2C.

3.2.12 Histological Studies

Animals were sacrificed as in above on post infection day 3, 7, 14 and 21 for sub-group 2A, 2B, 2C, 2D and group 1, respectively. Then, decapitation was performed and the brain tissues were carefully removed in whole, weighed and fixed in Bouin's fluid. The length, width and breadth parameters were taken after 1week of fixation to avoid autolysis.

Brain tissues were processed by, first, dehydrating in graded alcohol (30%, 50%, 70% , 95% and absolute alcohol), cleared in xylene and embedded in paraffin wax. Coronal and sagittal sections were taken with a rotary microtome (Leitz Wetzlar) at 10µm thickness for hippocampus and hypothalamus, respectively. Sections were floated in a water bath at 2°C below the melting point of the paraffin wax (65°C) and mounted on

glass slide using DPX. Sections were stained with routine heamatoxylin and eosin and cresylfast violet (Bancroft and Stevens, 2008). Immunohistochemistry staining was carried out with the aid of anti-calbindin antibody (Lee , 2008), following the protocol indicated in appendix vii.

3.2.13 Histomorphometric Study

Neuronal cells were counted using medium power field microscopy. Estimates of the number of pyramidal nerve cells were done by counting the number of the viable CA3 pyramidal cells in the hippocampus of each group tissue under a magnification of x250. This was done using the markers of cellular viability to distinguish between viable and death cells under a specific field (Olga, 2008). Pyramidal cells were identified on the basis of their position, orientation, shape, presence of an apical dendrite and prominent, single nucleolus .

Cellular measurements were done for both infected and uninfected groups using digimizer 2.2 software.

Statistical Analysis

Results were analysed using the statistical software, statistical package for social scientist (SPSS version 20.0) and expressed as Mean ±S.E.M. The presence of significant differences in means of the groups were determined using one way ANOVA with LSD post hoc test. Independent sample *t*-test were also employed for the comparisons of means as appropriate. Values were considered significant when $p \leq 0.05$.

CHAPTER FOUR

4.0 Results

4.1 Morphological Studies

Before inoculation, all the experimental animals (Wistar rats) were observed to exhibit normal physical activity. Infection with *T. brucei brucei* showed a significant decrease in the body weight of animals in group 1 (control) and group 2D (21 days post-infection) with *p* – values of 0.04 and 0.0002 respectively. However, rats in group 2A, 2B and 2C were not significantly affected by infection as at the time of sacrifice (**Table 4.1** and **Figure 4.1**).

4.2 Degree of Parasitaemia

Parasitaemia was first detected on day 3 post-infection with mean parasitaemia of (376.00±55.90 $x10^3$/ml of blood), level of parasitaemia incresed and reached a peak level of (33.41 ± 10.57x 10^7/ml of blood) on day 14 post-infection and this was significantly higher ($p<0.05$, $p<0.01$ and $p<0.001$). The parasitaemia levels then decreased (**Figure 4.2**). Thereafter, parasitaemia dropped and rose to another peak (333333.33 ± 210818.51 x 10^3/ml of blood) on day 16 post-infection. This peak was then followed by another drop in the number of detectable parasites before the termination of the experinment (**Figure 4.2**).

4.3 Haematological Studies

Results of haematological analysis demonstrated a significant($p \leq 0.05$) decrease in white blood cell count(WBC), red blood cell count(RBC), haemoglobin concentration(HGB), lymphocyte(LYM), and neutrophil(NEUT) count in all the infected groups compared to the control group(**Table 4.2 and 4.3**).

When the mean values of each parameter for all the animals were compared between pre-infection and post-infection, there was a significant decrease, at p-values: $p \leq 0.001$ for WBC, $p \leq 0.05$ for HGB, $p \leq 0.05$ for LYM and $p \leq 0.01$ for NEUT) using paired sample *t*-test for means as shown in Figure **4.3.**

4.4 Morris Water Maze Training and Test Result

At the end of the training, all the animals learnt how to locate the hidden platform, as it was reflected in their ability to locate the platform within a short time. The difference in the means time it took the rats to carry out the task was insignificant with *p* values greater than 0.05 (**Figure 4.4**).

Result showed that infection with *T. brucei brucei* induced latency deficiency in memory. There was a decrease in their ability to locate the hidden platform, as the time increased with the time course of the infection. With the exception group 2A and 2B, there was a significant increase in the latency time across the infected groups, when compared with control. Latency period increase was statistically significant in groups 2C and 2D with *p*-value: 0.000, compared to uninfected control. Rats in group 2D showed highest latency time to locate the platform (**Figure 4.5**).

Table 4.1.Weight Differences Between Control and Infected Groups

	Mean±Std. Error for Pre-infection	Mean±Std. Error for Post-infection
Control	214.50±38.05	227.50±35.89*
GP2A	215.83±27.91	218.67±26.59
GP2B	206.33±12.84	205.33±12.97
GP2C	206.00±8.33	199.20±9.97
GP2D	201.83±5.24	188.83±5.22***

N=6, values are means±SEM; During test, *= significant difference at p (<0.05); **= significant difference at p (<0.01); ***= significant difference at p (<0.001) when compared with control using LSD post hoc test. GP= Group

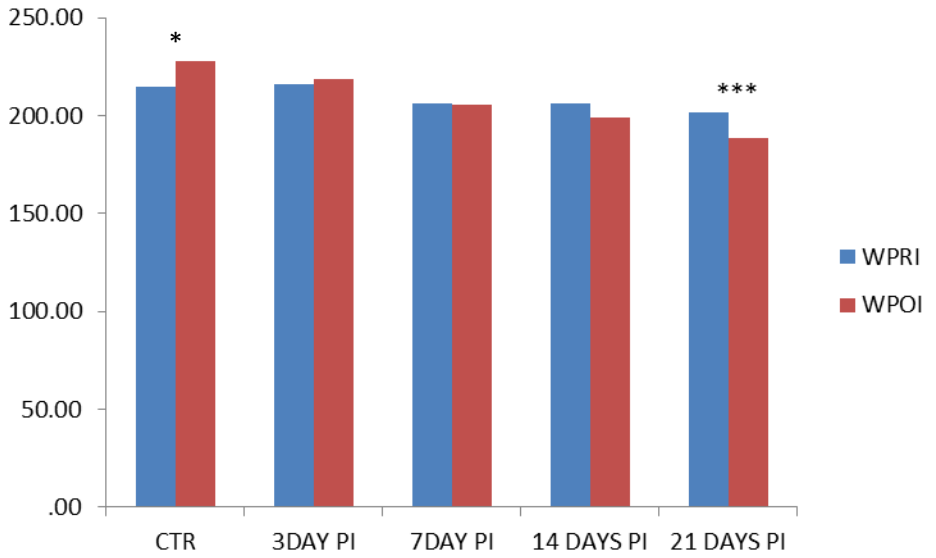


Figure 4.1: Weight comparison of experimental animals pre-innoculation and post-innoculation.

Paired sample *t*- test for means: During test, *= significant difference at *p* (<0.05); ***= significant difference at *p* (<0.001) using LSD post hoc test. N= 6. **CTR**= Control; **WPRI**= Weight pre-infection; **WPOI**= Weight post-infection; **PI**= Post-infection.

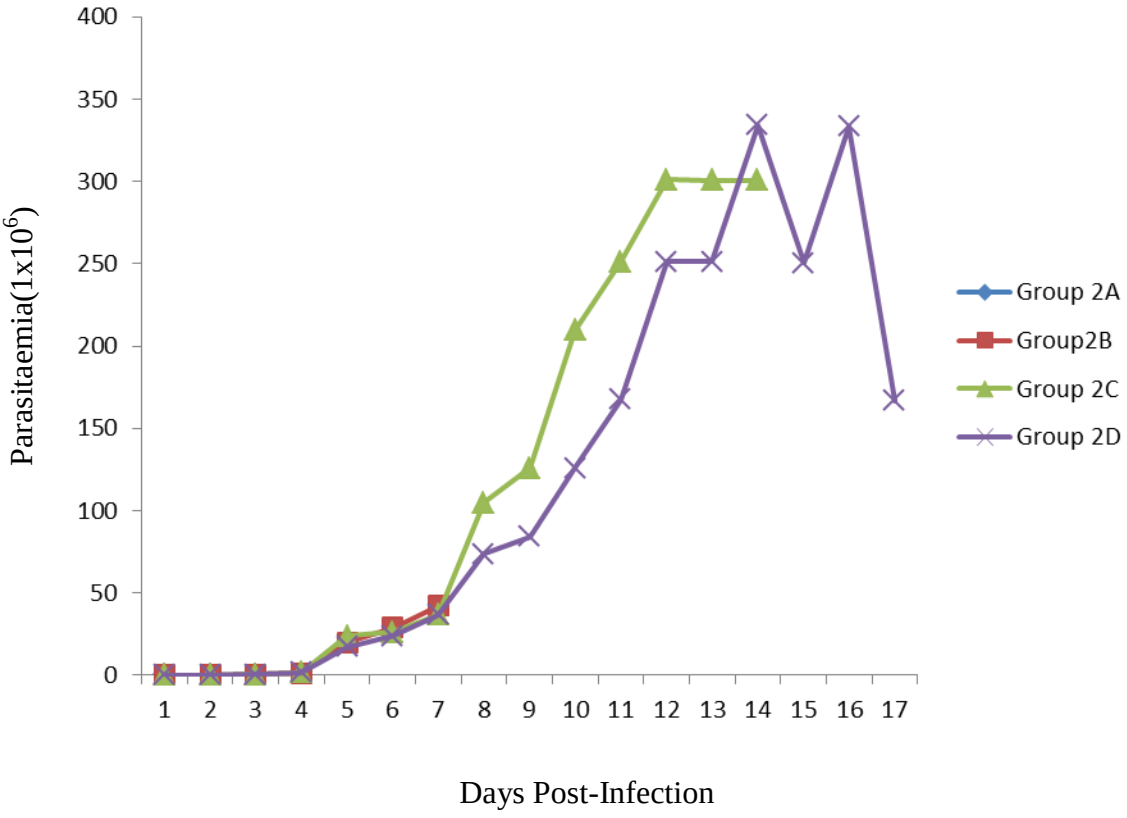


Figure: 4.2: Levels of parasitaemia across the groups.

Table 4.2: Blood Parameters Comparing Control and other Groups Before Infection

	Mean ± Std. Error				
Parameter	**Control**	**Group 2A**	**Group 2B**	**Group 2C**	**Group 2D**
WBC(x10^{3}uL)	17.53±1.69	18.82±2.50	13.96±0.76	10.74±0.39	10.02±1.74
RBC(x10^{6}uL)	8.51±0.19	8.51±0.24	8.75±0.37	9.57±0.69	7.84±0.08
HGB(g/dL)	15.34±0.40	15.22±0.24	15.72±0.69	12.47±0.83	14.80±0.20
LYM(%)	72.48±2.58	72.95±2.13	67.13±1.77	52.24±1.57	69.55±1.39
NEUT(%)	24.93±2.30	22.00±2.13	32.89±1.77	47.72±1.57	30.45±1.39

N=5, values are means ± SEM; a = significant difference at p=(≤0.05); b = significant difference at p=(≤0.01); c = significant difference at p=(≤0.001) when compared with control using LSD post hoc test. **WBC**= White blood cell; **RBC**= Red blood cell ; **HGB**= Haemoglobin; **LYM**= Lymphocytes; **NEUT**= Neutrophiles.

Table 4.3: Blood Parameters Comparing Control and other Groups After Infection

	Mean ± Std. Error				
Parameter	**Control**	**Group 2A**	**Group 2B**	**Group 2C**	**Group 2D**
WBC(x10^{3}uL)	10.10±1.72	9.43±1.99	5.47±0.17	4.98±0.03^b	4.81±0.01^b
RBC(x10^{6}uL)	7.68±0.23	7.20±0.48	4.65±0.32^c	3.10±0.19^c	4.69±0.01^c
HGB(g/dL)	13.47±0.26	12.97±0.81	8.52±0.40^c	5.70±0.19^c	8.04±0.06^c
LYM(%)	68.07±2.12	71.33±2.29	75.03±1.29b	76.01±0.04^c	75.01±0.05^b
NEUT(%)	31.93±2.12	28.17±2.00	19.98±1.29c	20.98±0.02^c	19.08±0.05^c

N=5, values are means ± SEM; a = significant difference at p=(≤0.05); b = significant difference at p=(≤0.01); c = significant difference at p=(≤0.001) when compared with control using LSD post hoc test. WBC= White blood cell; RBC= Red blood cell ; HGB= Haemoglobin; LYM= Lymphocytes; NEUT= Neutrophiles.

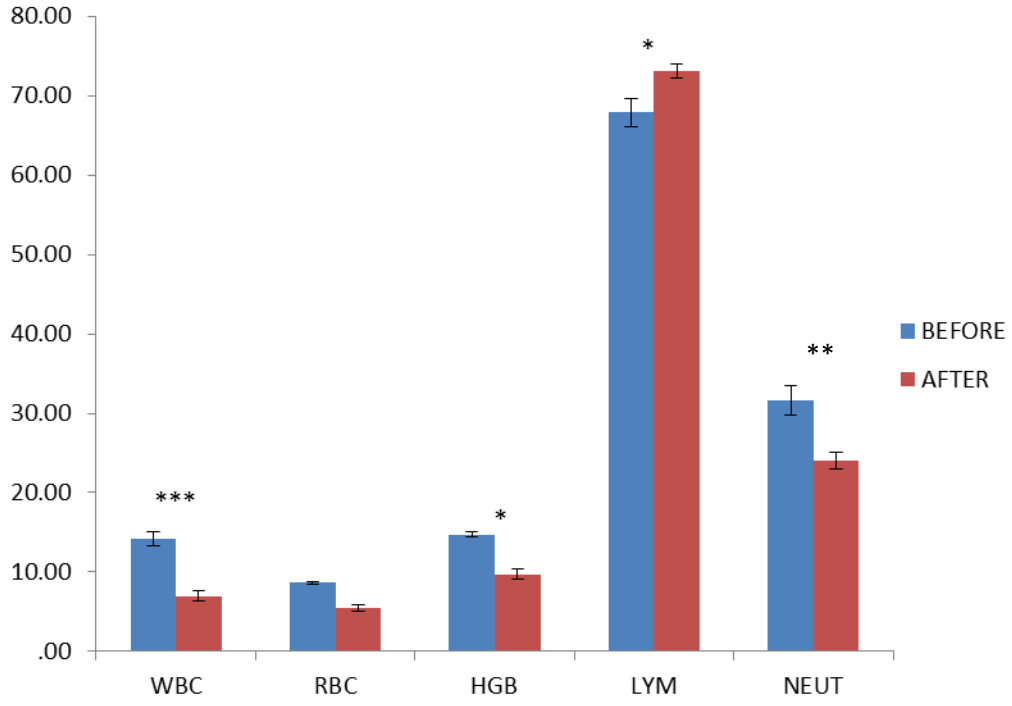


Fig 4.3: Blood parameter comparison of means of all animals before and after infection

Paired sample *t*-test for means: * = significant difference at $p=(\leq 0.05)$; ** = significant difference at p (< 0.01); *** = significant difference at p (< 0.001); n = 6. **WBC**= White blood cell ($x10^3$/uL); **RBC**= Red blood cell ($x10^6$/uL); **HGB**= Haemoglobin(g/dL); **LYM**= Lymphocytes (%); **NEUT**= Neutrophiles(%).

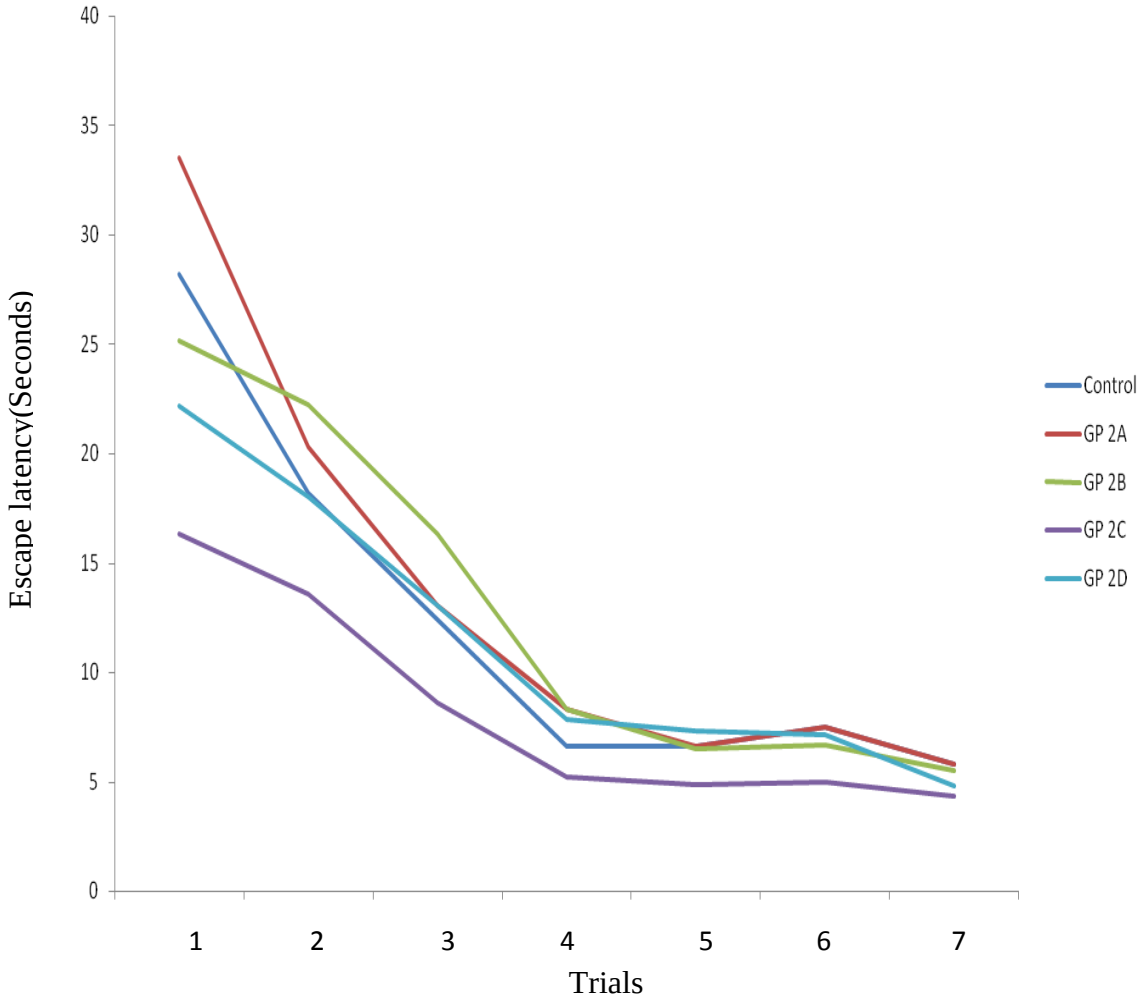


Figure 4.4: Latency to reach the hidden platform during training blocks of Morris water maze in different groups.

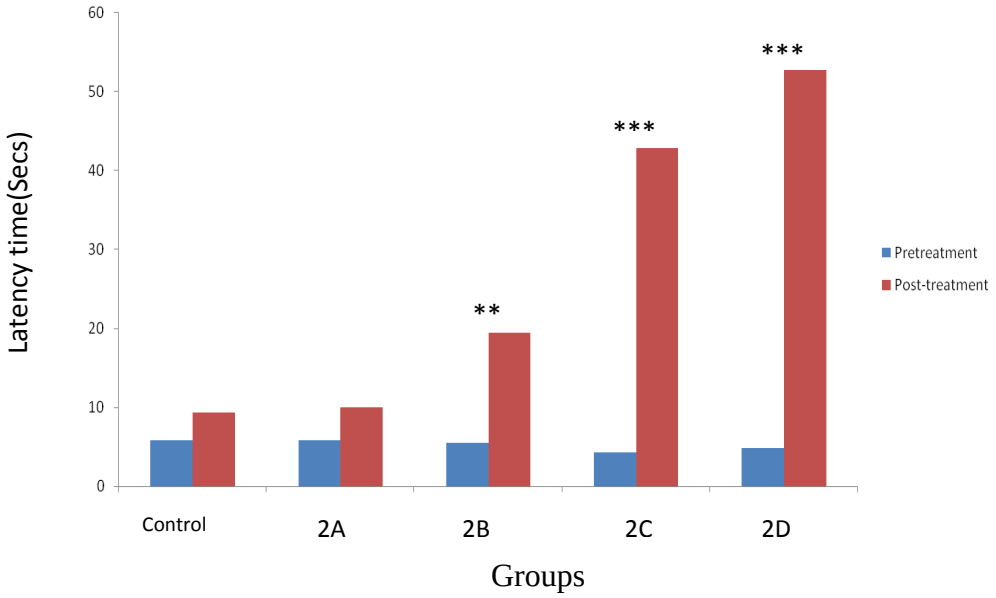


Figure 4.5: Comparing between the means training and test times of groups.

Paired sample *t*- test; * = significant difference at *p* (< 0.05); ** = significant difference at p (< 0.01); *** = significant difference at p (< 0.001);compared to control.

4.5 Morphometric Studies

There was a slight decrease in the length of the cerebrum when the control was compared with the infected groups, but reached significant level in group 2D was significant ($p<0.05$) (**Figure 4.6**). No significant difference in the cerebral width and breath between control and group 2A (3days post infection) but a highly significant decrease in the cerebral width and breadth ($p\leq0.001$) was observed between control and groups 2B, 2C and 2D. Infection with *T. brucei brucei* induced changes in the cerebral dimensions (**Figure 4.6**).

A decrease was recorded when the body weight and brain weight of the control was compared with that of the infected group (**Figure 4. 7**), though the decrease in body weight was not significant. The decrease in brain weight was very significant ($p=0.05$ with group 2B and $p<0.001$ with group 2C and 2D). When the brain to body weight ratio was taken, it showed no significant difference when that of the control was compared with other groups (**Figure 4. 7**).

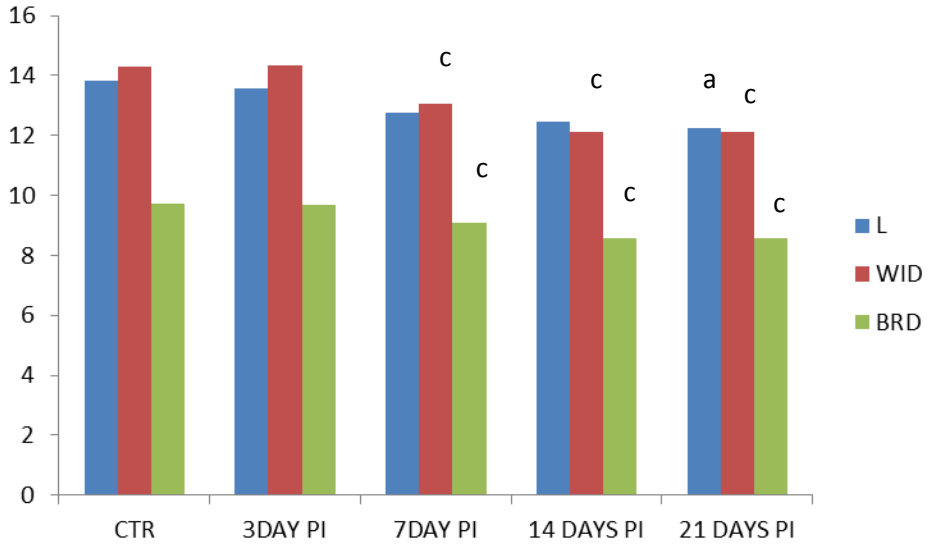


Fig 4.6: Comparing cerebral Length, Width and Breadth between Uninfected and Infected groups

N=5, values are means ± SEM; a = significant difference at p=(≤0.05); b = significant difference at p=(≤0.01); c = significant difference at p=(≤0.001) when compared with control using LSD post hoc test. CTR= Control; PI= Post-infection; L= Length; W= Width; BRD= Breadth.

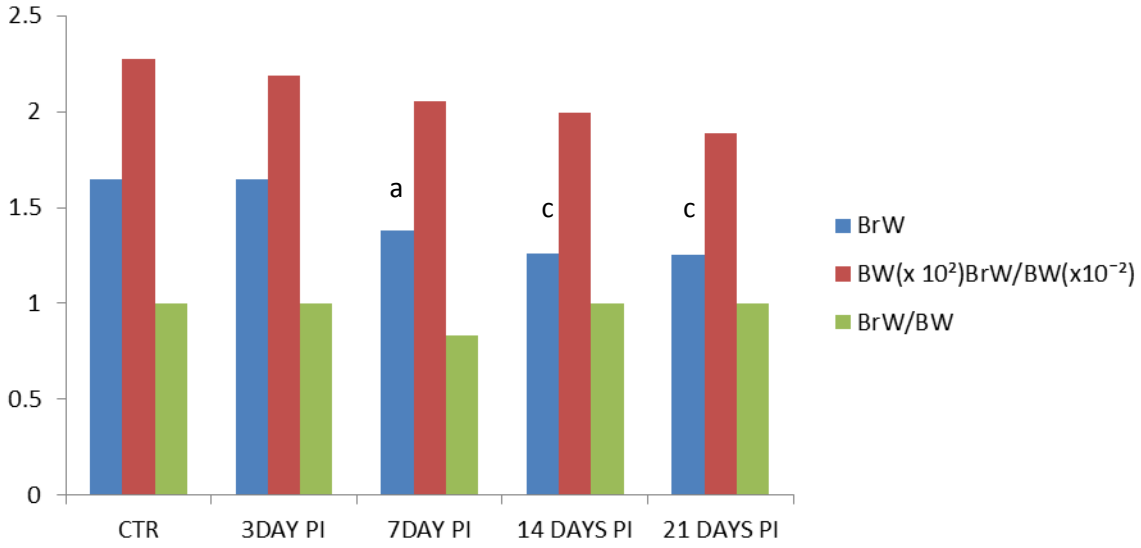


Fig 4.7: Comparing the Brain weight, Body weight and Brain-body weight ratio between Uninfected and Infected groups

N=5, values are means ± SEM; a = significant difference at p=(≤0.05); b = significant difference at p=(≤0.01); c = significant difference at p=(≤0.001) when compared with control using LSD post hoc test. CTR= Control; PI= Post-infection; Brw= Brain weight, Bw= Body weight.

4.6 Histological Study

Generally, inflammation was observed in the cerebral tissue of the infected animals, this was characterised by the high infiltration of lymphocytes in the infected cerebral tissue (**Plate 4.2**) and also, the appearance of morular cells. These were not seen in the control cerebral tissues (**Plate 4.1**). Also, molecular layer showed increase in glial cells, particularly, oligodendrocytes.

4.6.1 Histological Features of Hypothalamus and Hippocampus in both the Control (Uninfected) and Infected Experimental Animals

Sections from control rats showed normal architecture of the hypothalamus. This is characterised by the numerous giant cells that are cris-crossed by nerve fibers (**Plate 4.3**). However, sections from rats in group 2D showed that infection with *T. bricei brucei* markedly affected the histoarchitecture of the hypothalamus. This was characterised by the infiltration of inflammatory cells and few giant cells (**Plate 4.4**).

Futhermore, the histology of the hippocampus showed marked changes in group 2D rats. This was in the form of cell loss of pyramidal cell, especially, in the CA3 region of which had pale nuclie, while others showed dark nuclei. Distorted cellular arraingement of the hippocampal region, this is depicted by the spreading and non-larminar arrangement of the cells, (**Plate 4.6**), this is against the normal larminar arraingement of that of the control (**Plate 4.5**). Majority of the pyramidal cells in the CA3 region are not viable as they either don't have nucleus or it has become very faint (**Plate 4.8**).

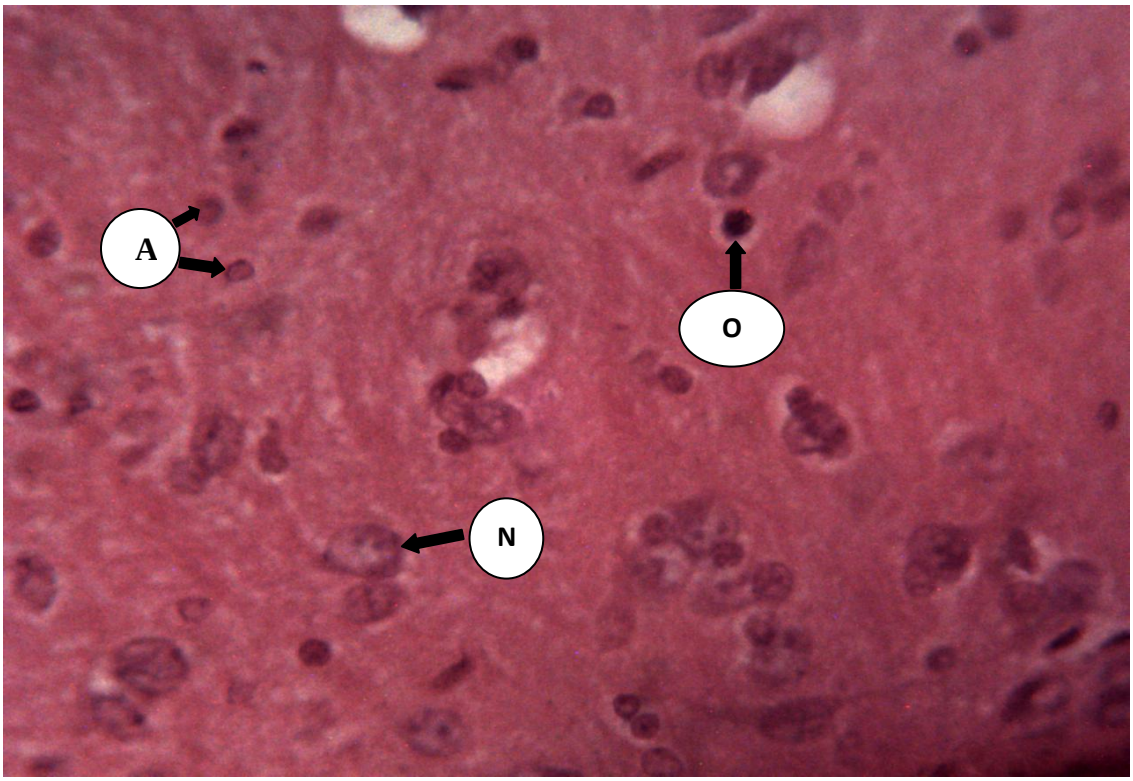


Plate 4.1: Section of the cerebral hemisphere of the normal (uninfected) experimental animal (Wistar rat) showing normal histology of the cerebrum: Astrocytes (A); Neuron(N); Oligodendrocyte(O). H and E stain (Mag x 400).

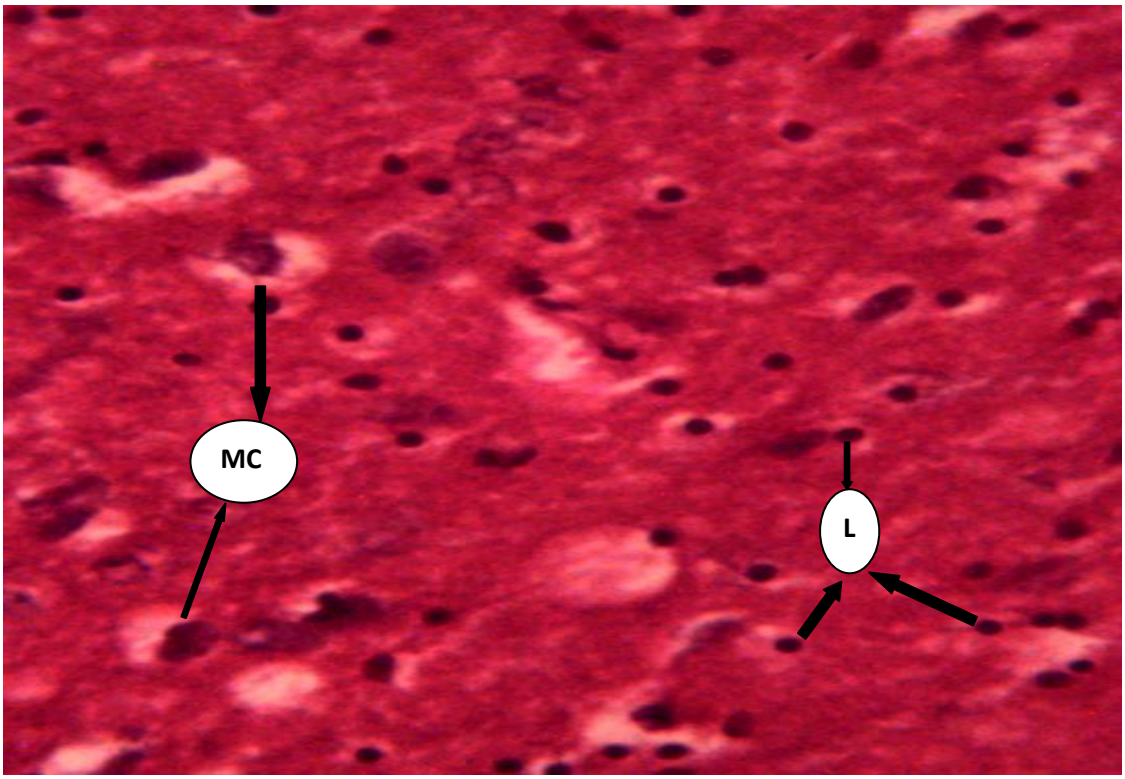


Plate 4.2: Section of the cerebral hemisphere of Group 2D (infected) experimental animal (Wistar rat) showing the presence of Morular cells (MC) very high appearance of Lymphocytes (L). H and E stain (Mag x 400).

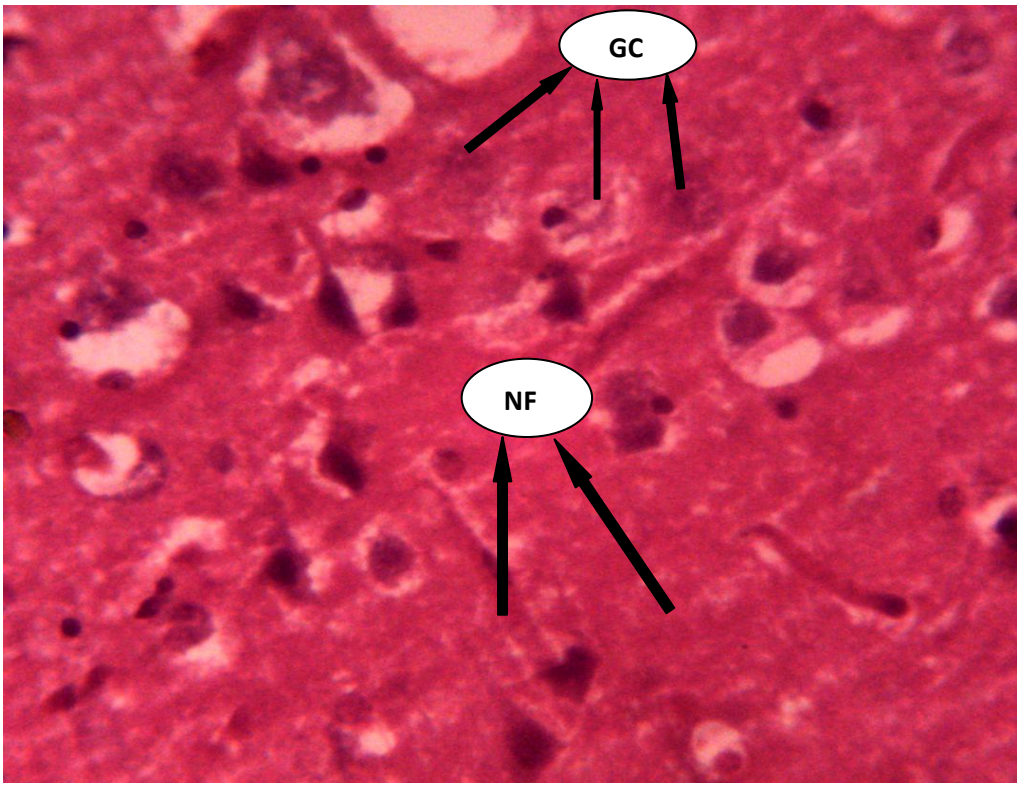


Plate 4.3: Section of the hypothalamus of the normal (uninfected) experimental animal (Wistar rat) showing normal histology: This is characterised by the numerous giant cells(GC) that are cris-crossed by nerve fibers(NF). H and E stain (Mag x 400).

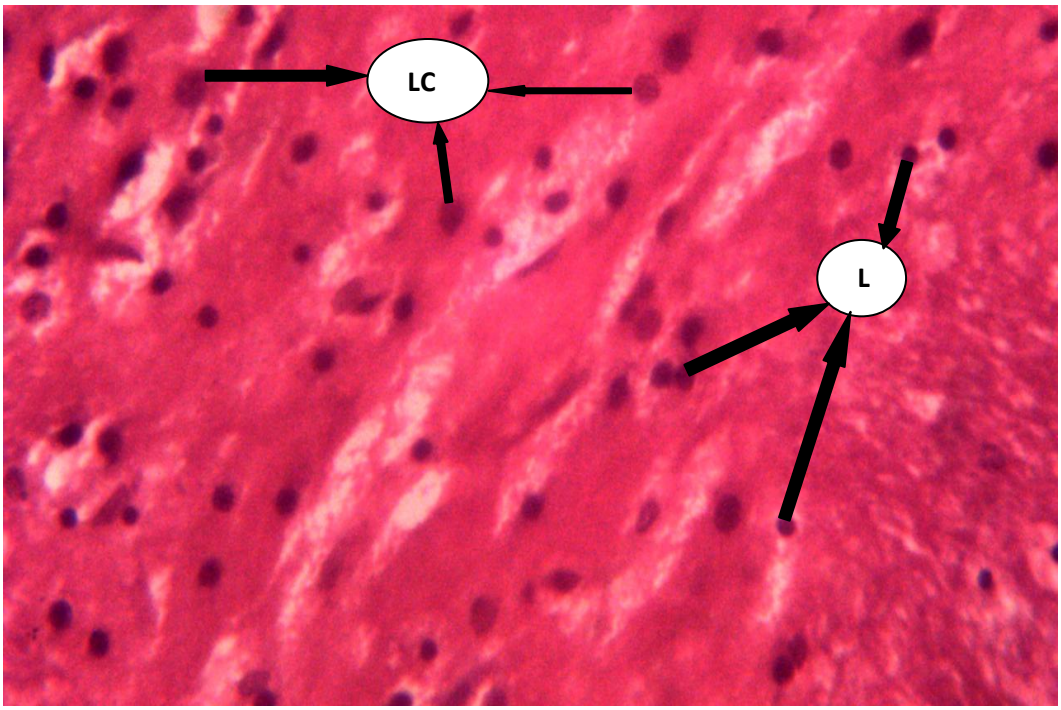


Plate 4.4: Section of the hypothalamus of Group 2D (infected) experimental animal (Wistar rat) showing very high appearance of Lymphocyte(L) and few large cells(LC). H and E stain (Mag x 400).

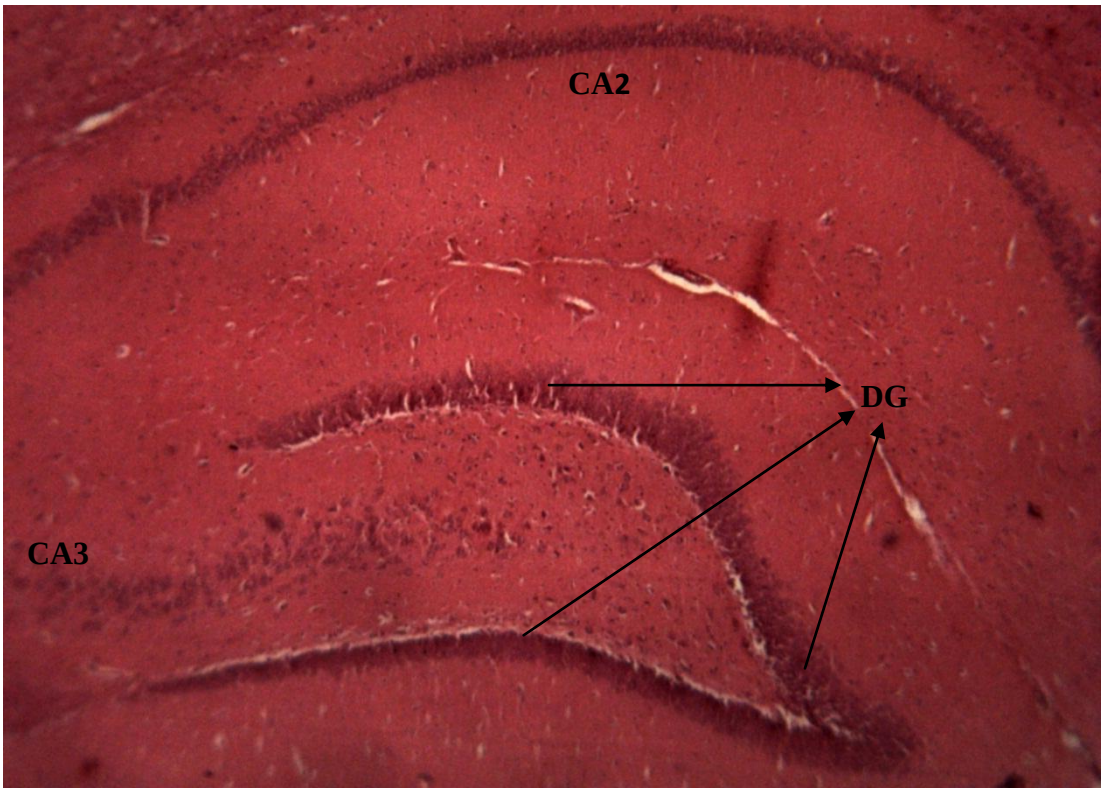


Plate 4.5: Section of the hippocampal formation (hippocampus) of the control (uninfected) experimental animal (Wistar rat) showing normal histology of the hippocampus: Dentate gyrus (DG); Cornud ammorni (CA). H and E stain (Mag x 40).

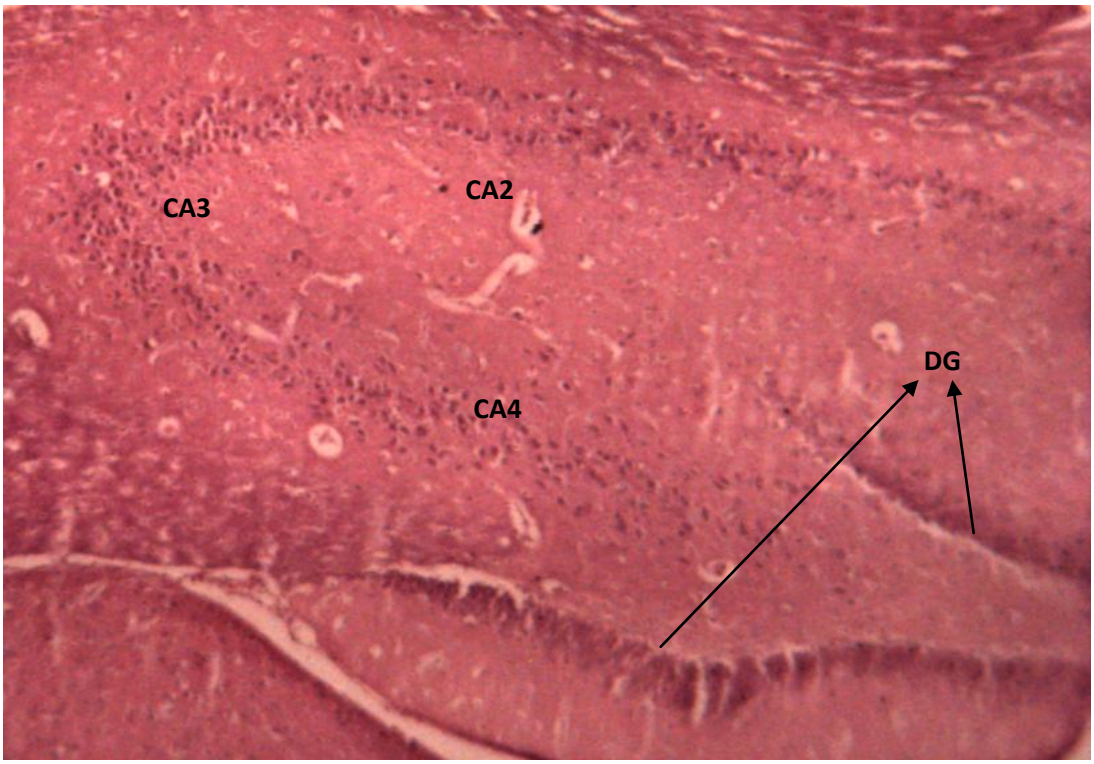


Plate 4.6: Section of the hippocampal formation (hippocampus) of group 2D (infected) experimental animal (Wistar rat) showing loosed arraingement of the CA3 and CA4 cells: Dentate gyrus (DG); Cornus ammorni (CA). H and E stain (Mag x 40).

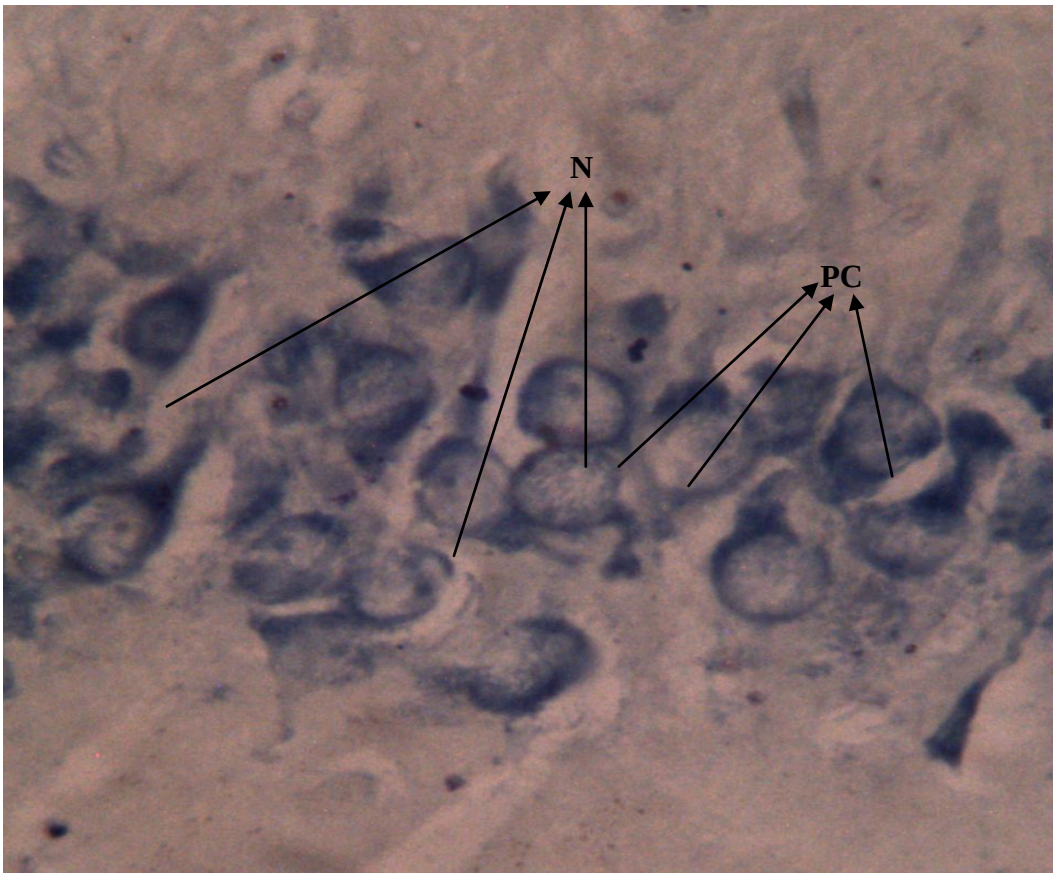


Plate 4.7: Section of the hippocampus (CA3) of control (uninfected) experimental animal (Wistar rat) showing normal histology of the cells and their nucleus: Pyramidal cells (PC); Nucleus (N). Toulidine blue stain (Mag x 400).

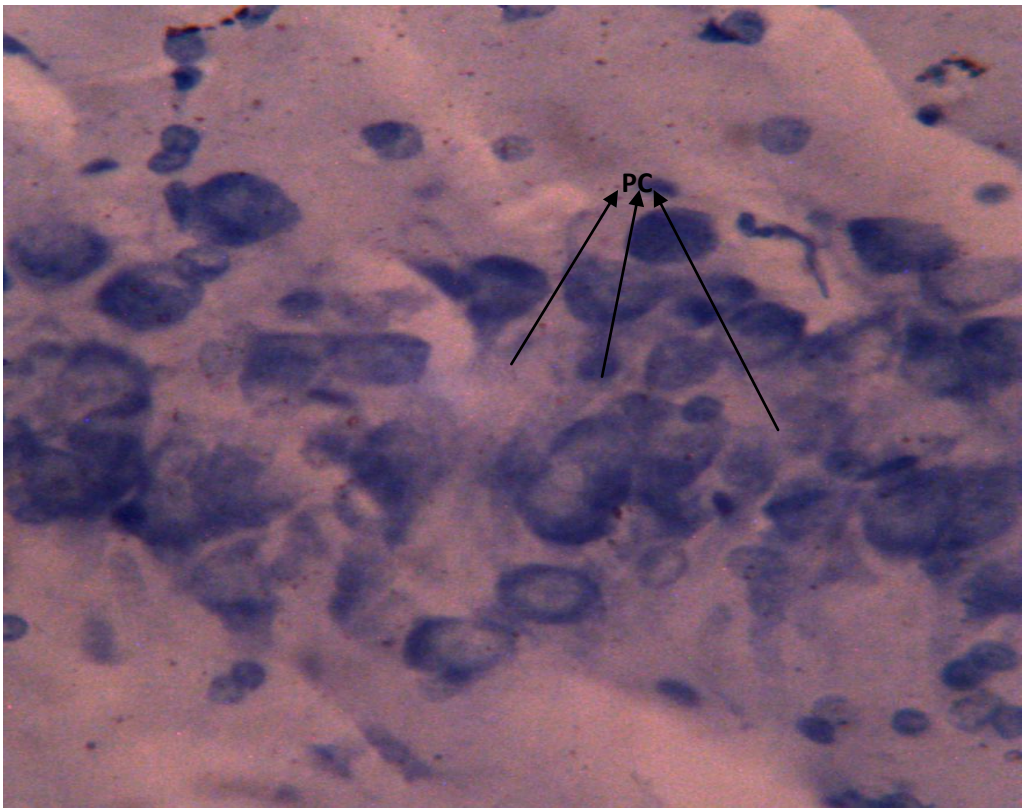


Plate 4.8: Section of the hippocampus (CA3) of group 2D (infected) experimental animal (Wistar rat) showing cells with faint or no nucleus: Pyramidal cells (PC); Nucleus (N). Toulidine blue stain (Mag x 400).

4.7 Histomorphometrical Studies

The number of cells in the CA3 region decreased across the groups with significant difference (p<0.001) only in group 2C and 2D, when compared to the control (**Table 4.4**). The diameter and area of cells also decreased across the groups with significant difference (p<0.001) in group 2B, 2C and 2D, when compared to the control (**Table 4.4**).

4.8 Immunohistochemical Studies

The immunohistochemistry stain of the cerebral hemisphere showed more rings of calbindin d28k in the control (**Plate 4.9**) compared to infected group (**Plate 4.10**). This difference was also seen in the CA3 region of the hippocampus (**Plate 4.11 and 4.12**).

Table 4.4. Comparing the Number of Cells, Cell Diameter, Cell Area of both Control and Infected Group.

GROUPS	CELL COUNT	DIAMETER	AREA
CONTROL	58.83±1.30	15.55±0.15	190.00±3.71
2A	56.83±1.68	15.40±0.14	186.42±3.50
2B	56.67±1.63	13.85±0.22***	150.88±4.86***
2C	23.50±0.85***	11.56±0.34***	105.37±6.19***
2D	15.33±0.99***	9.67±0.23***	73.79±3.46***

N=5, values are means ± SEM; * = significant difference at p=(≤0.05); ** = significant difference at p=(≤0.01); ***= significant difference at p=(≤0.001) when compared with control using LSD post hoc test. WBC= White blood cell; RBC= Red blood cell ; HGB= Haemoglobin; LYM= Lymphocytes; NEUT= Neutrophiles.

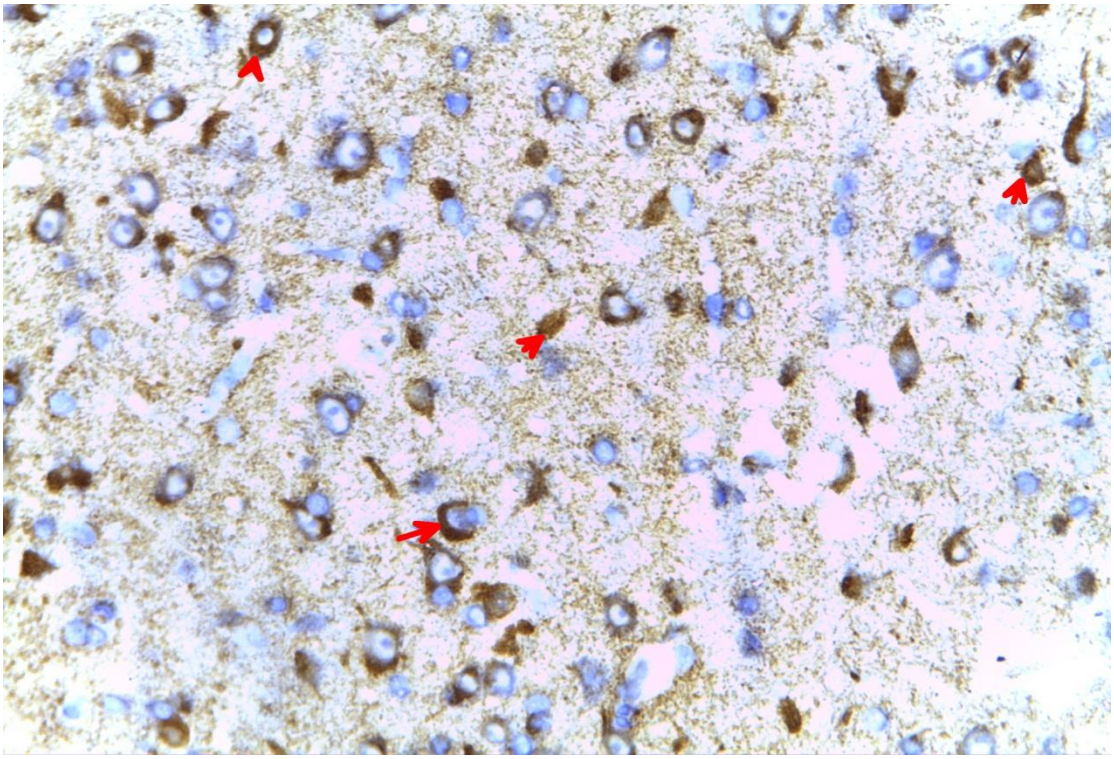


Plate 4.9: Section of the Cerebral hemisphere of group 1 (uninfected) experimental animal (Wistar rat) showing more rings of calbindin d28kcells: Immunohistochemistry stain (Mag x 400).

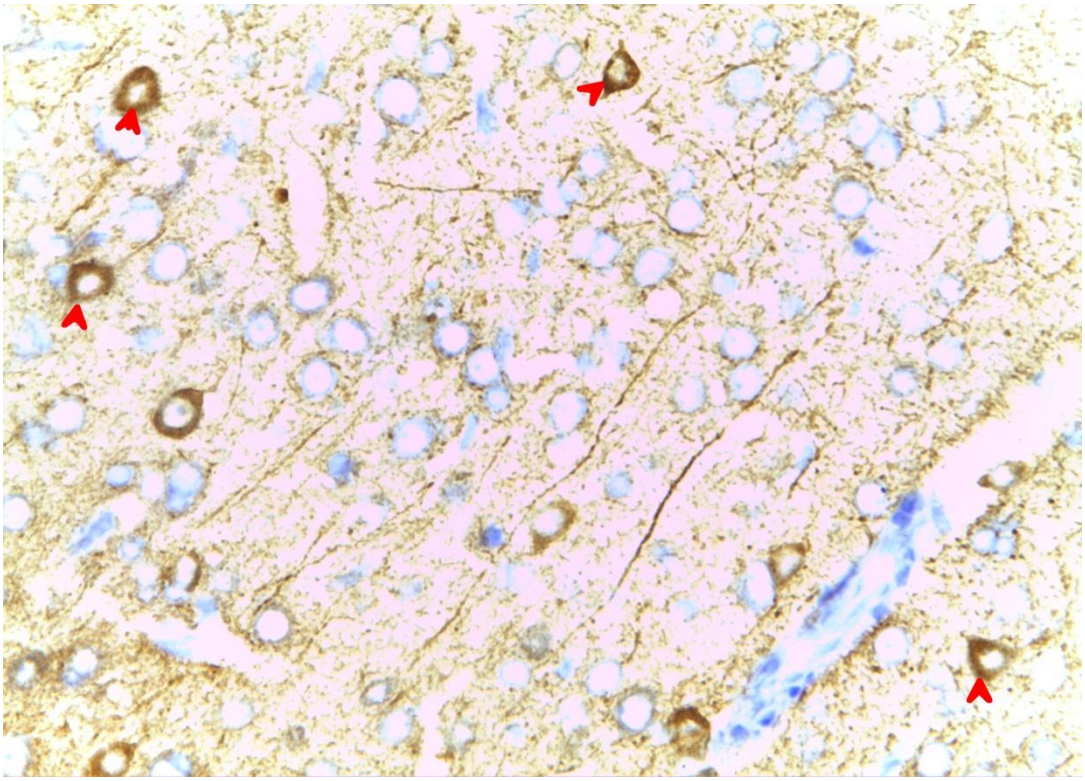


Plate 4.10: Section of the Cerebral hemisphere of group 2D (infected) experimental animal (Wistar rat) showing few rings of calbindin d28kcells: Immunohistochemistry stain (Mag x 400).

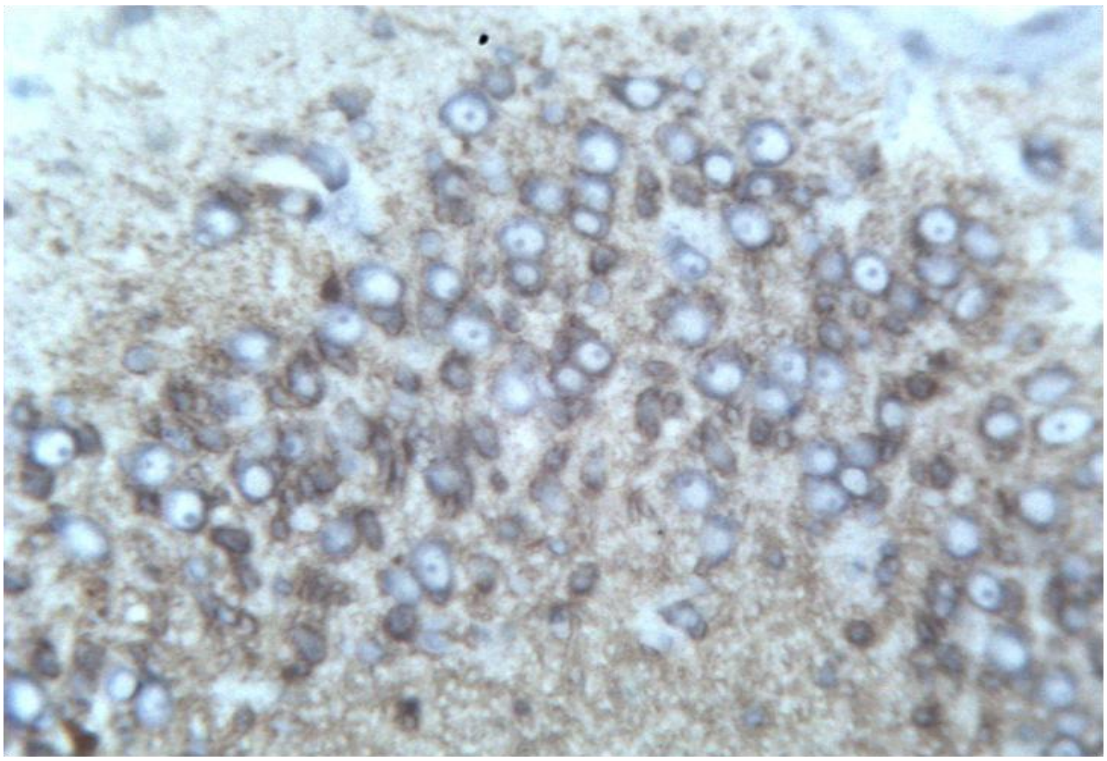


Plate 4.11: Section of the CA3 region of the hippocampus of group 1 (uninfected) experimental animal (Wistar rat) showing more rings of calbindin d28kcells: Immunohistochemistry stain (Mag x 250).

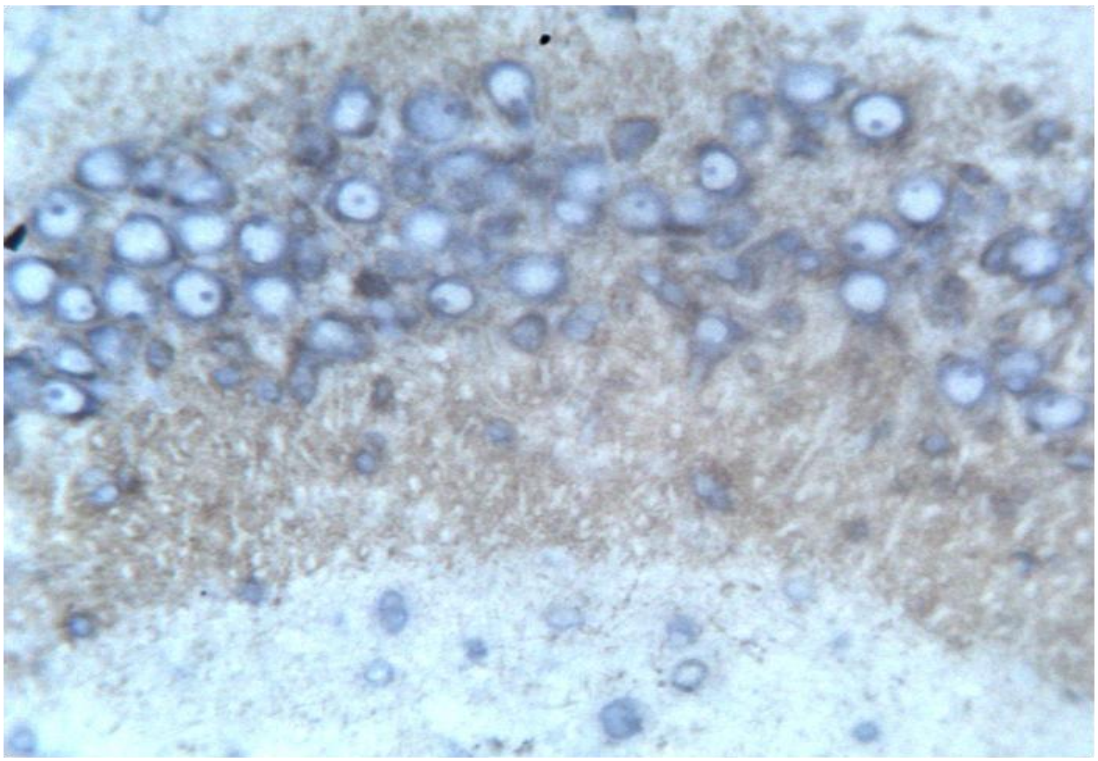


Plate 4.12: Section of the CA3 region of the hippocampus of group 2D (infected) experimental animal (Wistar rat) showing fewer rings of calbindin d28kcells: Immunohistochemistry stain (Mag x 250).

CHAPTER FIVE

5.0 Discussion

5.1 Mophorlogical Studies

Physical observation was in form of the condition of the rats after infection. Infected rats showed gain in body weight from the beginning of the experiment to about the 7th day after infection, this was followed by a continuous decrease in body weight as against weight gain in normal rats; this disagrees with the report of Humphrey *et al*, 1997, which showed a continuous increase in body weight throughout the period of the experiment. However,the result of this study agrees with the report of Adenowo *et al*, (2004); they recorded a prominent decrease in body weight throughout the experiment (Ohaeri, 2010). Better growth rate observed in uninfected rats could be attributed to increased food and water consumption and weight loss in infected rats could be attributable to parasite-induced lack of appetite and muscular degeneration. Itard, (1990) stated that the apparent weight loss was due to increase in parasitaemia. Changes in weight might possibly reflect the severity of the disease progress and also serve as a diagnostic aid. It has also been shown that nutritional status of the host influences the pathogenesis of the infection (Agyemang *et al* , 1990; Little *et al* , 1990) by promoting body weight gains; therefore animals should constantly be well nourished so as to withstand parasitaemia.

The pathologic substrate of late-stage sleeping sickness is meningoencephalitis in which cellular proliferation occurs in the leptomeninges, and a diffuse perivascular white matter infiltration consisting of lymphocytes, plasma cells, and macrophages is prominent (Atouguia and Kennedy, 2000, Adams *et al*, 1986). Extensive oedema, infiltration of inflammatory cells and rupture of ventricular ependymal layer are found are also

associated with late stage of trypanosomiasis (Atouguia and Kennedy, 2000). All these are expected to increase the size of the brain, but the result of this research does not support that as it showed a significant reduction in the mean weight, length, breadth and width.

5.2 Parasitaemia Observation

The present study demonstrates that *T. brucei brucei* when inoculated into rats develop parasitaemia within three days, this is in agreement Ajagbonna *et al (2005*)., Anene *et al*, (2006), Ameh *et al*, (2006) and Haruna et al (2013). In contrast, the first parasitaemia record of *Trypanosoma congolense*, occurs in days 4 and 7 post infection respectively (Habila *et al*, 2012). This suggests that *T. brucei brucei* is more virulent. The study showed that parasitaemia reached its peak (334133.33 ± 105662.24x 103/ml of blood) on the 14th day post-infection; and disagree with that recorded by Haruna *et al* (2013) which reported peak parasitaemia within 5-6 days with Trypanosoma brucei brucei, but it agrees with the findings of Habila *et al* (2012) and Ohaeri (2010) for Trypanosoma congolense, who reported peak values (341.22 x 106/ml of blood) and (337 ± 1.2 x 106/ml of blood) respectively on the 16th day post-infection. Thereafter, parasitaemia dropped and rose to another peak (333333.33 ± 210818.51x 103/ml of blood) on day 16 post-infection, which was then followed by another fall in parasitaemia; this is also similar to the report of Ohaeri (2010).

5.3 Hematological Study

The appearance of the parasites in the blood corresponds to a period of clinical situation that was associated with the continuous decrease in WBC, RBC, HGB, total neutrophils and lymphocytes of animals across the groups. The marked decrease of RBC counts

indicates anaemia (Umar *et al*., 2010 and , Habila *et al*, 2012). This is in line with the report of Murray (1979), who stated that the onset and severity of anaemia in trypanosomiasis is directly related to the appearance of the parasite in blood and the level of parasitaemia. The decrease in RBC indicates disorders in the blood due to *T. brucei brucei* parasites and results in decrease in immune function, increase in neurological disorder and lymphocytopenia(Gadelha *et al*., 2011). Pathogenesis of anaemia in trypanosomal infection seems to be variable in nature , but oxidative stress, also known to play a significant role in the pathogenesis of *T. brucei brucei* infection(Umar *et al.*, 2010). Haemodilution, osmotic and mechanical fragility of RBCs, extravascular haemolysis, decrease life span of RBCs and dyshaemopoiesis, all have been cited by various research workers as pathogenic mechanisms in different animals (Clarkson, 1968; Jatkar and Purohit, 1971; Anosa and Isoun, 1976; Dargie *et al*., 1979; Murray, 1979; Cox and Sale, 1983; Singh and Mishra, 1986). The haemodilution was also seen in this study, which is the only possible reason why the automated haematological analyser found it tough to analyse the most infected blood samples. The fall in WBC and lymphocyte counts agrees with the report of Blom-Potar *et al*, 2010. The decrease in neutrophil count is in contrast to past findings of, which reported its increase after infection (Padmaja, 2012).

5.4 Neurobehavioural Study

Before Infection, all the animals learnt how to locate the hidden platform, as it was reflected in their ability to locate the platform within a short time. After Infection, there was a decrease in their ability to locate the hidden platform, as the time taken to do that increases across the time course of the infection. This may indicates possible

neurodegeneration of brain areas responsible for learning and memory. The Morris water maze test was first established by neuroscientist Richard G. Morris in 1981 in order to test hippocampal-dependent learning, including acquisition of spatial memory and long-term spatial memory.

5.5 Histological Study

Lower levels of IFN- are found in the brain of mice during the early CNS stage of the disease compared with the late CNS stage animals (Sternberg *et al*., 2005).The presence of morular (Mott's cells) indicates a neurological disorder, as they contain immunoglobulins of the IgM class (Tarlinton *et al*, 1992). These cells are seen frequently in lymphoid tissues of murine and human autoimmune diseases (Schweitzer et al, 1991; Shultz *et al*, 1987). They have been found to occur in multiple myeloma (Weinstein *et al*, 1987), and have been observed also in late stages of African trypanosomiasis (Rozman et al, 1986).

The infected group shows disorganization and disintegration of the packed nature of the hippocampus, especially around the CA3 area. This has been reported as a sign of neurodegeneration (Amin *et al*., 2013). Marked shrinkage in size of large pyramidal cells, affecting outer layer more, with paler or no nuclei indicate cell death or apoptotic cells respectively. These deleterious effect on the hippocampus could result in deficit in memory and learning, loss of attention and distractibility, which have been reported to be associate with the disease (Kennedy, 2005).

The inflammatory cells observed in the cerebral tissue of the infected animals as against their absence in the control group depicts the early CNS stage of the disease. This

corresponds to past report, (Rodgers *et al.* 2009), it showed that animals exhibited early CNS stage disease showed only mild inflammatory changes with the presence of a limited number of T-cells and macrophages in the brain accompanied by a diffuse astrogliosis whereas late CNS stage mice show higher numbers of T-cell and macrophages as well as the presence of B-cells and plasma cells in the inflammatory infiltrate and a more pronounced astroglial activation (Jennings *et al*., 1997; Rodgers *et al* 2009).

5.6 Histomophormetrical Studies

The significant increase in length, width and breadth shown across the groups when compared to the control is not in line with the reports of Atouguia and Kennedy, 2000; and Adams *et al*, 1986. They reported meningoencephalities due to cellular proliferation in the leptomeninges in late-stage sleeping sickness. The difference in the current research could be due to the difference in the length of time of exposure to the parasite, which in their case, it was for a longer perion. The brain-body weight index decreased from the unset and later began to increase; this is in line with theeir reports.

5.7 Immunohistochemical Studies

There was a decrease in the expression of calbindin d28k in the infected group when compared to the control. This was indicated by more of the calbindin rings in the uninfected groups than in the infected, which were much more. This result corroborate the significant increase in the latency time recorded during the Morris water maze test. This is in line with the report of Lee *et al*. (2009).

CHAPTER SIX

6.0 Summary, Conclusion and Recommendation

6.1 Summary

The study demonstrated that Trypanosoma brucei brucei infection significantly affected the blood parameters, it also affected the histology, histomorphometrics, histochemistry and the expression of calbindin d28k. These effects led to reduction in spartial memory and learning.

6.2 Conclusion

The result of the present study demonstrated that, *T. brucei brucei* infection significantly induced parasitaemia which significantly affected the blood parameters. It also affected spartial memory which was corroburated by its effects on histology, histomorphometrics, histochemistry and the expression of calbindin d28k.

6.3 Recommendation

- More prolonged studies should be carried out in order to show the progression of the infection in the brain.
- Other structures in the brain should be studied in relation to trypanosomiasis.
- Neuronal tracing should be attempted to see if it can explain the possibility of any effect of the parasite on neuronal synaptic connection.
- Immunohistochemical studies to determine the nature of neurons involved in trypanosomiasis should be looked at.

APPENDIX I

COMPOSITION OF PELLITIZED FEED FROM GRAND CEREALS AND OIL MILLS LIMITED, JOS, NIGERIA.

Crude Protein	14.50%
Fat	7.00%
Crude Fibre	7.20%
Calcium	0.80%
Available Phosphorous	0.40%

APPENDIX II

CALCULATIONS OF THE INNOCULATION DOSE

The donor animal had an in fulminating parasitemia of 64 per field.

64 per field ≡ 251,200,000 parasite/ml

1ml of the blood was diluted with normal saline to give 1parasite per field

→1per field ≡ 3,943,000 = 4,000,000

Therefore, 1ml = 4,000,000

This was futher diluted by 3ml of normal saline

→4ml = 4,000,000

Therefore, 1ml = 1,000,000 parasite

This implies that, an innoculum dose of 1 x 106 trypanosomes per 1ml volume prepared in normal saline was used to infect the rats in group 2 via intraperitoneal(IP) route, while the group 1 rats were given 1ml of normal saline intraperitoneally(Ohaeri, 2010).

APPENDIX III

TISSUE PROCESSING

1. **Fixation** in Bouin's fluid.
2. **Washing** in running water for 2 hours.
3. **Dehydration** in grades of alcohol

- 30% alcohol for 4 hours
- 50% alcohol for 4 hours
- 70% alcohol for 6 hours
- First change 95% alcohol for 4 hours
- Second change 95% alcohol for 4 hours

4. Xylene + absolute alcohol equal parts for 2-4 hours
5. **Clearing**

- Put in pure xylene first change for 4 hours
- Pure xylene second change for 4 hours

6. **Infiltration**

- Xylene + paraffin 2-4 hours
- Pure paraffin 16 hours

7. **Embed** in paraffin

APPENDIX IV

PREPARATION OF HAEMATOXYLIN AND EOSIN STAINING SOLUTION

1. HAEMATOXYLIN

- Haematoxylin -1g
- Absolute alcohol -10ml
- Ammonium or potassium alum -20g
- Distilled water -200ml
- Mercuric oxide -0.5g

Heamatoxylin was dissolved in alcohol and alum which was dissolved in water was added. The mixture was then boiled and mercuric acid added when solution turn dark purple, it was then cooled rapidly.

2. Eosin

- Distilled water
- Crystal of thymol

5% eosin solution was made by dissolving 5g of eosin in 100ml of distilled water. To prevent growth of mould in the stock solution, a crystal of thymol was added to obtain 1% staining solution, 20ml of the stock solution was diluted by making it up to 100ml.

3. STAINING

- **Removal of wax** with xylene

➢ Dewaxing I, 3 minutes

➢ Dewaxing II, 3 minutes

- **Hydration** with graded alcohol
  - Absolute alcohol, 1 minute
  - 95% alcohol, 1 minute
  - 70% alcohol, 2 minutes
  - 50% alcohol, 2 minutes
  - 30% alcohol, 1 minute
- **Staining**
  - Haematoxylin, 10 – 20 minutes
  - Distilled water (washing)
  - 35% alcohol, 1 minute
  - Acid-alcohol, 30 seconds (for differentiation between nucleus and cytoplasm)
  - Distilled water, 1 minute
  - Alkaline-alcohol, 2 minutes (for bluing of the nucleus)
  - Distilled water, 1 minute
  - Staining in 1% eosin, 2 minutes
- **Dehydration** in alcohol
  - 90% alcohol, 10 – 15 seconds
  - Absolute alcohol, 1 minute
- **Cleaning**
  - Xylene-alcohol, 2 minutes
  - Pure xylene I, 3 minutes
  - Pure xylene II, 3 minutes
- **Mounted** in DPX

APPENDIX V

IMMUNOHISTOCHEMISTRY STAINING PROTOCOL FOR CALBINDIN D28K

- Dewax and hydrate
- Wash in distilled water
- Retrieve antigen in 0.1molar citrate solution (PH 9)
- Wash in PBS (PH 7.4)
- Apply peroxidase block (5mins)
- Wash in PBS
- Wash in protein block (5mins)
- Wash in PBS
- Apply primary antibody (anti-calbindin) for 1hr
- Wash in PBS
- Apply post primary antibody
- Wash in PBS
- Apply polymer (30mins)
- Wash in PBS
- Apply constituted DAP (5mins)

- Wash in PBS
- Counterstain with heamatoxylene (1min)
- Dehydrate, clear and mount in DPX

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