THE EFFECT OF CIGARETTE SMOKE MAINTAINED AT 10 – 32 PART PER MILLION OF CARBON MONOXIDE ON THE HIPPOCAMPUS OF WISTAR RATS

BY

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

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NOVEMBER, 2011.

DECLARATION

I, Badamasi Mohammed Ibrahim, hereby declare that the work in the thesis titled "The effect of cigarette smoke maintained at 10 – 32 part per million of carbon monoxide on the hippocampus of wistar rats" has been performed by me in the Department of Human Anatomy under the supervision of DR. S.S. Adebisi and DR. A.O. Ibegbu.

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.

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CERTIFICATION

This thesis entitled "The effect of cigarette smoke maintained at 10 – 32 part per million of carbon monoxide on the hippocampus of wistar rats'' by Badamasi Mohammed Ibrahim meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

THIS THESIS IS DEDICATED

to

MY PARENTS

And to

MY DEAR WIFE

And to

OUR CHILDREN

ACKNOWLEDGEMENTS

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ABSTRACT

Memory and Learning are diverse and complex entities attracting numerous studies with different findings, some even contrasting each other. This study was conducted to make a contribution towards unravelling this reality. Behavioural studies using the Morris Water Maze task was elicited in Wistar rats which were grouped for weight and sex (n^{male}=18; n^{female} =12). Reference or latency time recordings for three (3) training at the start of the experiment and after a 5week period of exposure to cigarette smoke (in case of exposed Rats) or 5weeks training free period (in case of non-exposed Rats). Analysis of the time recordings suggest that there is a significant decrease ($p \leq 0.05$)for the control male Wistar rats unlike the insignificant($p \geq 0.05$) change obtained for the control and experimental female and experimental male. Similarly, assessment of the histological section of the hippocampus of the Male Wistar rats from both tissue sections and hippocampal cell suspension was done. Cell counting of normal and Pyknotic pyramidal cells was done using Image J soft ware. Analysis of the cell count suggests a significantly higher ($p \leq 0.05$) count in the Exposed Males only, for both the normal and pyknotic pyramidal cells compared to the counts of the Control Male rats. In conclusion, male Wistar rats tend to retain Reference memory of Morris Water Maze Task for a longer time (5 weeks in this case) compared to female Wistar rats. Similarly, cigarette smoke maintained at 10-32ppm of Carbon Monoxide for 4 days in a week for 5 weeks suffices in disrupting the memory capabilities of the Wistar rats. With a corresponding level of higher rates of Pyknotic pyramidal cell.

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ABBREVIATIONS

Alpha – amino-3- hydroxy-5-methyl- 4-isoxazolepropionic acid ……..……. AMPA

Caffeic Acid phenethyl ester ..... CAPE

Calcium and Magnesium free Hanks balanced salt solution .....CMF-HBSS

Calcium ion ..... Ca^{2+}

Calcium- calmodulin dependent kinase II .....CaMKII

Carbon monoxide ..... CO

Carboxy- Hemoglobin ..... CO-Hb

Cornu Ammonis ..... CA

Deoxyribonucleic acid ..... DNA

Entorhinal Cortex ..... EC

Environmental tobacco smoke ..... ETS

Federal Trade Commission ..... FTC

Glutamate ..... GLU

Hanks balanced salt solution ..... HBSS

Hemeoxygenase ..... HO

(4-(2-Hydroxyethyl)-1- piperazineethanesulfonic acid ..... HEPES

Laboratory of the Government Chemist ..... LGC

Marsland and Glees ..... M&G

Messenger ribonucleic acid ..... mRNA

Morris time ..... MT

Morris water maze ..... MWM

Morris water maze task ..... MWMT

N-methyl D-Aspartate ..... NMDA

N-methyl D-Aspartate receptor ..... NMDAR/NR

Oxygen ..... O_2

Part per million ..... PPM

Post titanic potentiation ..... PTP

Potassium ion ..... K^+

Prefrontal cortex ..... PFC

Sodium ion ..... Na^+

Standard deviation ..... S.D

Synaptic re-entry reinforcement ..... SRR

Three dimension 3D

CHAPTER ONE

1.0 Introduction

1.1 Background

Memory is a complex, diverse and heterogeneous entity and in recent decades, it has become one of the principal pillars of a branch of science called cognitive neuroscience, an interdisciplinary link between cognitive psychology and neuroscience. In psychology, memory is an organism's ability to store, retain and recall information. Tulving and Craik (2000) define memory as 'the ability to recollect past events and to bring learned facts and ideas back to mind'. An adequate definition of memory must incorporate other aspects of this complex phenomenon including, both conscious and non-conscious aspects of memory. Memory is 'the demonstration that behaviour has been altered as a consequence of the previous storage of information at some point in time ranging from a few seconds to several decades'(Jonathan, 2002). For descriptive purpose, memory could be viewed from the perspectives of duration, temporal and information type.

The duration perspective has the following entities: Sensory memory, which is the memory that corresponds approximately to the initial 200 – 500 milliseconds after an item is perceived. The ability to look at an item and remember what it looked like with just a second of observation or memorization is an example of sensory memory (Sperling, 1960).

While, a short term memory is the memory that allows recall for a period of several seconds to a minute without rehearsal. Long-term memory can store much larger quantities of information for potentially unlimited duration sometimes a whole life span. The capacity can

also approach infinity (unlimited). Memory could be viewed from temporal direction of the information to be remembered, this addresses whether the content to be remembered is in the past, “retrospective memory”, or whether the content to be remembered is in the future, “prospective memory”. Retrospective memory includes semantic, episodic and autobiographical memory. In contrast, prospective memory can be divided into event- and time-based prospective remembering (Winograd, 1988). Finally is, the classification of memory based on information type, which divides long-term memory into declarative (explicit) and procedural (implicit) memories (Anderson, 1976). Procedural memory is primarily employed in learning motor skills and should be considered a subset of implicit memory since it is devoid of conscious recall of information. Procedural memory depends on the cerebellum and basal ganglia. Where a declarative memory requires conscious recall, it is sometimes called explicit memory, since it consists of information that is explicitly stored and retrieved. It can be further sub-divided into: Semantic memory, which concerns facts taken independent of context and Episodic memory, which concerns information specific to a particular context, such as a time and place. Autobiographical memory and spatial memory are subset of episodic memory (Conrad, 1964).

The hippocampus is hypothesized to be important in a memory system that participates in encoding relationships among stimuli (Cohen and Eichenbaum, 1993). Nearly all investigators agree that any event which significantly alters the function of the hippocampus, either temporarily or permanently will cause amnesia in humans and rats.

Well-studied examples include drug administration (Toumane and Derkin, 1993), electroconvulsive shock (Squire *et al*.,1981), brain trauma (Markowitsch *et al*., 1993), brain ischemia (Zola-Morgan., 1986), hypoxia (De Renzi and Lecchelli, 1993), encephalitis

(Yoneda *et al*., 1992), surgical excision of brain tissue (Corkin, 1984), and new learning (Keppel,1984). There are many reports that damage to the hippocampus has a detrimental effect on the recall of recent but not remote memories (Squire, 1992) amnesia. There are two important properties of memories which determine their vulnerability to hippocampal damage; Type and Age (Sutherland *et al*., 2001).

Regarding type of memory, spatial memory (Morris *et al*.,1990), conditioning to context (Kim *et al*., 1993) are affected following hippocampal damage while learning tasks which require navigation to a location marked by a single landmark, are spared (McDonald and White, 1993). The second property of memories which determines the vulnerability of memory to disruption by hippocampal damage is age. A commonly reported pattern of recall in retrograde amnesia following hippocampal damage is that remote events are remembered better than recent events. This pattern of recall has come to be known as temporally graded retrograde amnesia. These suggest that memories become more resistant to disruption by hippocampal damage or dysfunction as time passes after the learning episode. This transformation of memories from a labile to a stable form is termed memory consolidation. There are numerous reports of temporally graded retrograde amnesia lasting from a few months to decades as a result of damage to the hippocampus in humans (Larry *et al*., 1996). The well-studied patient Henry Molaison (H.M) exhibits temporally graded retrograde amnesia. He is unable to recall events which occurred during several year intervals prior to his surgery. More remote memories are apparently unaffected (Corkin, 1984).

In contrast, there are also many reports of retrograde amnesia which is not temporally graded (Kapur *et al*., 1992). Patients with retrograde amnesia that is more or less equivalent for all past events regardless of the time that the memory was encoded prior to hippocampal

damage are said to have a "flat gradient" (Sutherland *et al.,* 2001). The length of "recent" memory loss in retrograde amnesia is reported to be days to many weeks in non-humans. For example, memory seems to be fully consolidated after 5 days in the case of socially transmitted food preference, since hippocampal damage after this point does not affect performance (Winocur, 1990). However, hippocampal-dependent memory consolidation takes 4 weeks in classical fear conditioning (Kim and Fanselow, 1992) and several months in the case of place navigation tasks (Kubie *et al.,* 1999).

1.2 Statement Of The Problem

The gap in knowledge stems from the absence of data relating the changes in spatial memory tasks in mammals directly to anatomical changes in the hippocampus attributable to cigarette smoke exposure maintained at 10-32 ppm of carbon monoxide. The findings in this study will strengthen the scientific understanding of the toxicological effect of cigarette smoke on the hippocampus and try to extend and correlate the findings to the spatial memory function of the hippocampus.

1.3 Study Hypothesis

Hoa: The difference in the cell count of the cell suspension of the male hippocampus of the study groups is statistically significant.

Hia: The difference in the cell count of the cell suspension of the hippocampus of the study groups is statistically significant

Hob: The pre and post exposure latency time in the Morris Water Maze task for the study animals is not statistically significant.

Hib: The pre and post exposure latency time in the Morris Water Maze task for the study animals is statistically significant.

Hoc: The difference in the cell count of the CA1 and CA2 regions of the hippocampus of the study groups is not statistically significant

Hic: The difference in the cell count of the CA1 and CA2 regions of the hippocampus of the study groups is statistically significant

1.4 Justification

In smokers and patients suffering from a variety of neuropsychiatric disorders, nicotinic agonists act beneficially on several aspects of cognition, including working memory attention, learning and memory (Levin *et al*., 2005). In contrast, in normal nonsmokers, nicotine tends to have deleterious effects on cognitive performance (Newhouse *et al.*, 2004b). On the other hand, adolescent nicotine treatment evoked decreases in total cell number as assessed by DNA measurements in cerebral cortex, midbrain and hippocampus (Trauth *et al*., 2000). Likewise, Zoli *et al*. (1999) showed that stimulation of nicotinic cholinergic receptors in mature cells can actually decrease the cell death elicited by injurious treatments potentially by induction of neurotrophic factors. On the other hand, Olcay *et al.,* (2007) showed an increase in apoptotic bodies in the hippocampus of rabbits that were exposed to the cigarette smoke. There is a need for a study of 10-32 PPM (low concentration) of carbon monoxide, with adequate counting of pyramidal cell in the

hippocampus and relating the count to memory capacity of Wistar rats in Morris water maze task.

1.5 Aim

To determine the structural and functional effects of exposure to cigarette smoke maintained at 10-32 PPM of carbon monoxide on the hippocampus of Wistar rats.

1.6 Objectives

(1)To compare the memory ability of the rats using records of time from Morris water maze task before and after exposure to cigarette smoke

(2)To compare the number of pyramidal cells in the sections of the hippocampus of the male rats, between the normal and those exposed to cigarette smoke.

(3)To compare the number of pyramidal cells in the hippocampus cell suspension of the male Wistar rats between the normal and those exposed to cigarette smoke

CHAPTER TWO

2.0 Literature Review

2.1 Anatomy of Hippocampus

The hippocampus is a sausage shaped brain structure which lies under the medial temporal lobe, one on each side of the brain. It can be divided into a dorsal portion (where it begins to bend ventrally and laterally) and a ventral portion lying in the temporal part of the brain. The part of the hippocampus visible on its dorsal aspect is the hippocampus proper, while the fascia dentate is buried inside and on the bottom surface of the sausage (O'Keefe and Nadel, 1978).The hippocampus proper is further divided on basis of cell morphology and fiber projection into region - superior and region – inferior respectively (Cajal, 1911). The region superior abuts on the prosubiculum and contain a double row of medium sized pyramidal cells, whose main apical dendrites gives off only small side branches and does not divide for several microns.

The region inferior is the semicircle close to the fascia dentate containing the giant pyramidal cells whose apical dendrites bifurcate shortly after leaving the soma. Lorente De Nó, (1934) divided the hippocampus proper into four (4) areas: Cornu ammonis (CA) 1- 4. He called Region superior CA1 and subdivided Region inferior into CA2 and CA3 and designated CA4 to the scattered cells inside the hilus of the fascia dentate. The CA4 area is rich with cells that have pyramidal cell like characteristics and receive mossy fibers, but unlike the CA3 pyramidal cells, they do not get fibers from basket cells. Hippocampal formation is a term referring to: hippocampus proper, Dentate gyrus, pre-subiculum, pro-subiculum and subiculum collectively (Amaral *et al*., 2006).

Signal flow through hippocampus and adjacent structures shows a mapping that resembles a loop. External inputs come from the adjoining entorhinal cortex (EC) through axons of perforant path. These axons arise from the second layer of entorhinal cortex and terminate in the third (3^{rd}) Cornu ammonis (CA3) and Dentate gyrus (DG). Another pathway from the third layer of EC to CA1, while Mossy fibers from granule cells terminate in pyramidal cells in CA3 and CA1. The interconnections described above are collectively called tri-synaptic circuit by Andersen (1976), who noted that thin slices could be cut out of the hippocampus perpendicular to its long axis in a way that preserves all of these connections and this serves as the basis of the lamellar hypothesis which proposed that the hippocampus is organized as a series of parallel strips operating in a functionally independent way. Recent modification to the lamellar hypothesis followed the discovery of extensive longitudinal connections within the hippocampal system (HS).

Moreover, each region of the HS contains a variety of inhibitory inter-neurons that in conjunction with principal cells give rise to a well-defined feedback and feed forward inhibitory local circuits (Andersen *et al.,* 2000). Plates 2.1 and 2.2 shows the gross and the histologic section of the hippocampus of Wistar rats respectively.

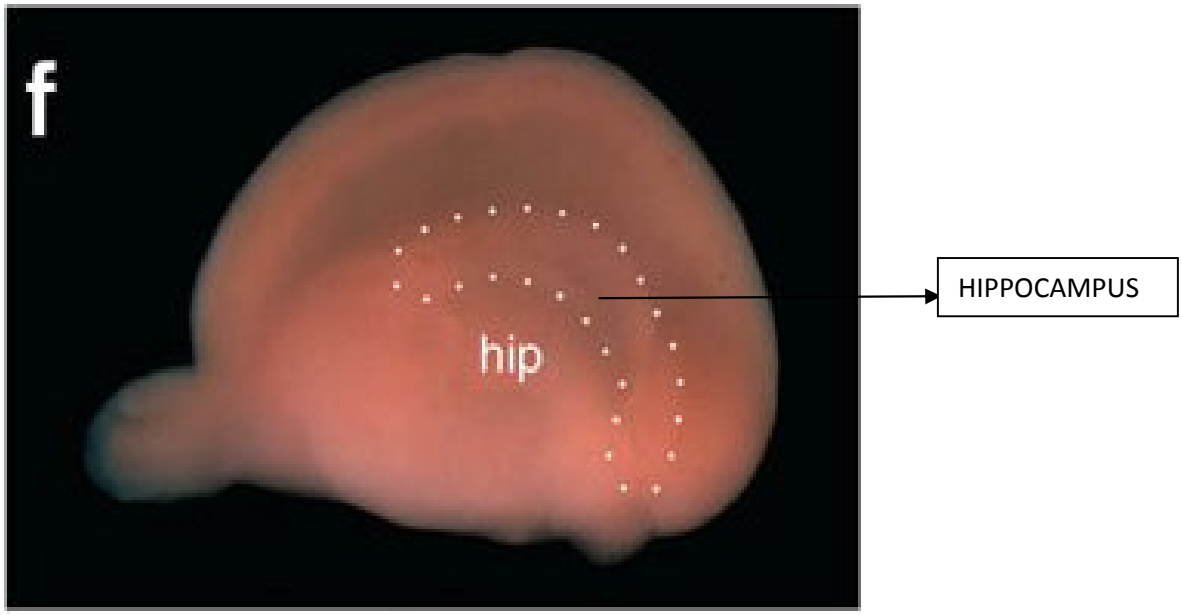

Plate 2.1: Gross Location of The Hippocampus (Source: Thomas *et al.,* 2008)

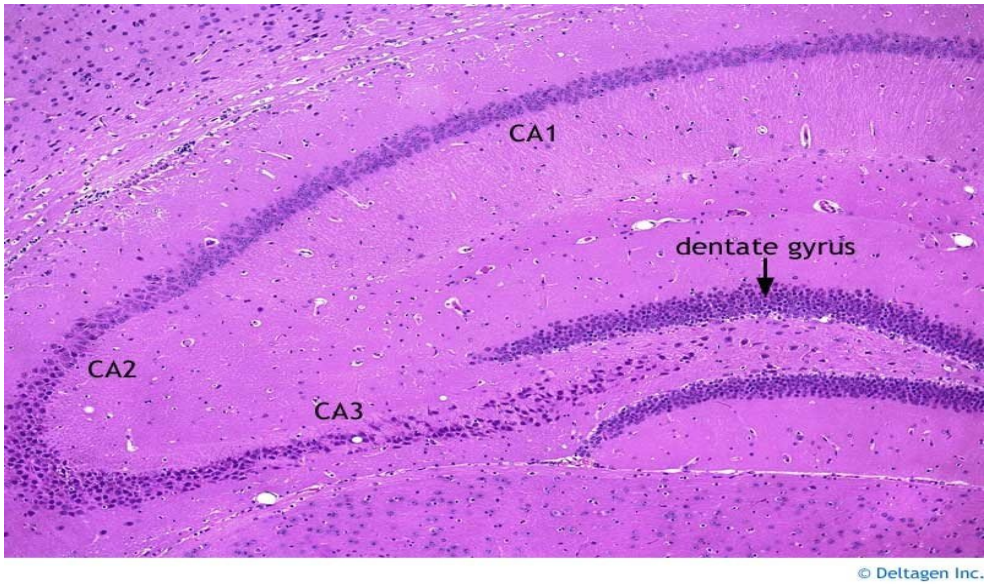

Plate 2.2: Section of Hippocampus (Source : Delta base [1M] Histology Atlas).

2.2 Place cells of the hippocampus

Neuroscientist generally agree that an intact circuit of the hippocampus and the hippocampus in general has an important role in the formation of memories and only more recently has it become clear that, within this role, only particular aspects of memory processing depend on hippocampal system function and that these properties can be distinguished operationally from other aspects of memory that do not depend on the hippocampal system; experienced events episodic or autobiographical memory(Squire *et al*., 2002). Part of this role is hippocampal involvement in the detection of novel events, places and stimuli (Van Elzakker *et al*., 2008). The pyramidal cells in the hippocampus proper and the granule cells of the dentate gyrus are specifically attributed with showing place responses and are called place cells. 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 and 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 to, as getting lost is one of the most common symptoms of amnesia (Chiu *et al*., 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 (Morris *et al*., 1982). The place cells are typically almost silent when a rat is moving around outside the place field, but they reach sustained rates as high as 40 Hz when the rat is near the center.

Neural activity sampled from 30–40 randomly chosen place cells carries enough information to allow a rat's location to be reconstructed with high confidence. This finding buttresses the significance of the place cells and hippocampus in spatial memory (Moser, 2008).

2.3 Memory and Spatial memory

In psychology, memory is an organism's ability to store, retain, and recall information. Tulving and Craik (2000) define memory as 'the ability to recollect past events and tobring learned facts and ideas back to mind'. An adequate definition of memory must incorporate other aspects of this complex phenomenon, including both conscious and non-conscious aspects of memory. It is 'the demonstration that behaviour has been altered as a consequence of the previous storage of information at some point in time ranging from a few seconds to several decades' (Jonathan, 2002). What is this spatial learning and memory? In their natural environment, some animals navigate over long distances – from summer to winter, to and from their breeding grounds, to their home loft from a distant release point, if they are homing or racing pigeons. Within their home range, which may be as small as a few square metres or as large as several hundred square kilometres, animals must find their way around, learn what the limits of that range are, who occupies neighbouring ranges, the way to food and water and the way back home, where they have stored food for later consumption, where predators may lurk, and where are suitable sites for a new home. And they must remember this information for days or months (Mackintosh, 2002).

Within the laboratory, rats have been trained to run through mazes to a single goal box, or to find the single piece of food at the end of each of eight or more maze arms radiating out from a central platform; they have learned to find food buried in the sand in one corner of a

rectangular arena, and to swim to submerged platform in a circular pool; in operant chambers they have been required to remember the location where a stimulus appeared a few seconds or minutes ago (Mackintosh, 2002). These spatial memory are declarative, which requires conscious recall, it is sometimes called explicit memory, since it consists of information that is explicitly stored and retrieved, it can be further sub-divided into; semantic memory, which concerns facts taken independent of context and episodic memory, which concerns information specific to a particular context, such as a time and place (Conrad, 1964). Spatial memory is a type of episodic memory. The other spectrum of memory in the context of the declarative memory isprocedural memory which is an entity of the long term memory just like the declarative memory and both are types of memory that are reflecting the type of information memorized. Procedural memory is primarily employed in learning motor skills and should be considered a subset of implicit memory since it is devoid of conscious recall of information (Conrad, 1964). It is revealed when one does better in a given task due only to repetition. Procedural memory depends on the cerebellum and basal ganglia.

Other types of memories include: "retrospective memory" the content to be remembered is in the past or "prospective memory" the content to be remembered is in the future. Retrospective memory includes semantic, episodic and autobiographical memory. In contrast, prospective memory can be divided into event and time-based prospective remembering, in this regard, spatial memory is a retrospective memory (Winograd, 1988). There is another type of memory that takes cognisance of the duration of the memory to be recalled. This include long-term memory which can store much larger quantities of information for potentially unlimited duration, sometimes a whole life span. The capacity

can also approach infinity (unlimited). For example, given a random seven-digit number, we may remember it for only a few seconds before forgetting, suggesting it was stored in our short-term memory. On the other hand, we can remember telephone numbers for many years through repetition; this information is said to be stored in long-term memory. While memory that corresponds approximately to the initial 200 – 500 milliseconds after an item is perceived is called sensory memory. Example is the ability to look at an item and remember what it looked like with just a second of observation or memorization (Sperling, 1960). And finally memory that allows recall for a period of several seconds to a minute without rehearsal is called short-term memory. Its capacity is also very limited as depicted in the work of George A. Miller in 1956 at Bell Laboratories which showed that the store of short term memory was 7±2 items in his famous paper, "The magical number 7±2". However modern estimates of the capacity of short-term memory are lower, typically on the order of 4-5 items (Cowan, 2001) and new findings show that memory capacity can be increased through a process called chunking. For example, in recalling a 10-digit telephone number a person could chunk the digits into three groups: first, the area code (such as 215), then a three-digit chunk (123) and lastly a four-digit chunk (4567). Short-term memory is believed to rely mostly on an acoustic code for storing information, and to a lesser extent a visual code (Conrad, 1964). In conclusion, it is worthy of note to say that spatial memory is a type of episodic memory of the retrospective type of the long term memory variety.

2.4 Memory Systems and Models

Interestingly, scientists have continually developed models that try to explain different types of memory and provide abstract representations of how memory is believed to work. Below are some models proposed over the years by various psychologists:

2.4.1 Atkinson-Shiffrin Model

The multi-store model (also known as Atkinson-Shiffrin memory model) was first recognised in 1968 by Atkinson and Shiffrin. The multi-store model has been criticized for being too simplistic. Long-term memory (LTM) is believed to be made up of subcomponents, such as episodic and procedural memory, similarly short-term memory can be divided into units e.g visual and acoustic information. Atkinson-Shiffrin formulated these models in the image of the behavioral characteristics of a particular patient, KF. The patient KF was brain damaged and had problems with his short term memory including spoken numbers, letters and words and with significant sounds (such as doorbells).Other parts of short term memory (STM) were unaffected like visual (pictures) (Cowan, 2001). It also proposed that rehearsal is the only mechanism by which information eventually reaches long-term storage but evidence of remembering things without rehearsal are numerous. It also shows the sensory store as a single unit whilst the sensory store is split up into several different parts such as taste, vision, and hearing. Figure 2.1 shows the multi store model of Atkinson and Shiffrin

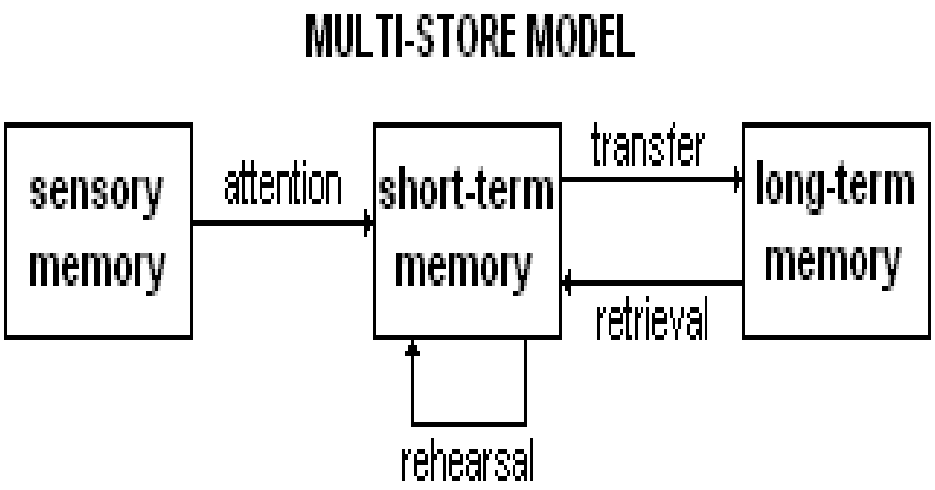

Figure 2.1: Multi store model of Atkinson and Shiffrin (Source: Atkinson and Shiffrin model of 1968

2.4.2 Working Memory Model

In 1974 Baddeley and Hitch proposed a working memory model as an alternative to the short-term store in Atkinson and Shiffrin's 'multi-store' memory model of1968. In this model, working memory consists of three basic stores: the central executive, the phonological loop and the visuo-spatial sketchpad. The 'central executive which acts as supervisory system and controls the flow of information from and to its 'slave-systems' the 'phonological loop' and the 'visuo-spatial sketch-pad'. The slave systems are short-term storage systems dedicated to a content domain (verbal and visuo-spatial, respectively). Baddeley and Hitch's (1974) argument for the distinction of the two domain-specific slave systems in the model was derived from experimental findings with dual-task paradigms. Performance of two simultaneous tasks requiring the use of two separate perceptual domains (a visual and a verbal task) is nearly as efficient as performance of the tasks individually. In contrast, when a person tries to carry out two tasks simultaneously that use the same perceptual domain, performance is less efficient than when performing the tasks individually. In 2000, Baddeley added a fourth component to the model, called the 'episodic buffer'. This component is a third storage system, dedicated to linking information across domains to form integrated units of visual, spatial and verbal information (e.g., the memory of a story or a movie scene) (Baddeley, 2000). The episodic buffer is also assumed to have links to the long term memory (LTM). The main motivation for introducing this component was the observation that some, in particular highly intelligent patients with amnesia, who presumably have no ability to encode new information in LTM, nevertheless have good STM recall of stories, recalling more than could be held in the phonological loop (Baddeley, 2000). Figure 2.2 shows the working memory model's components.

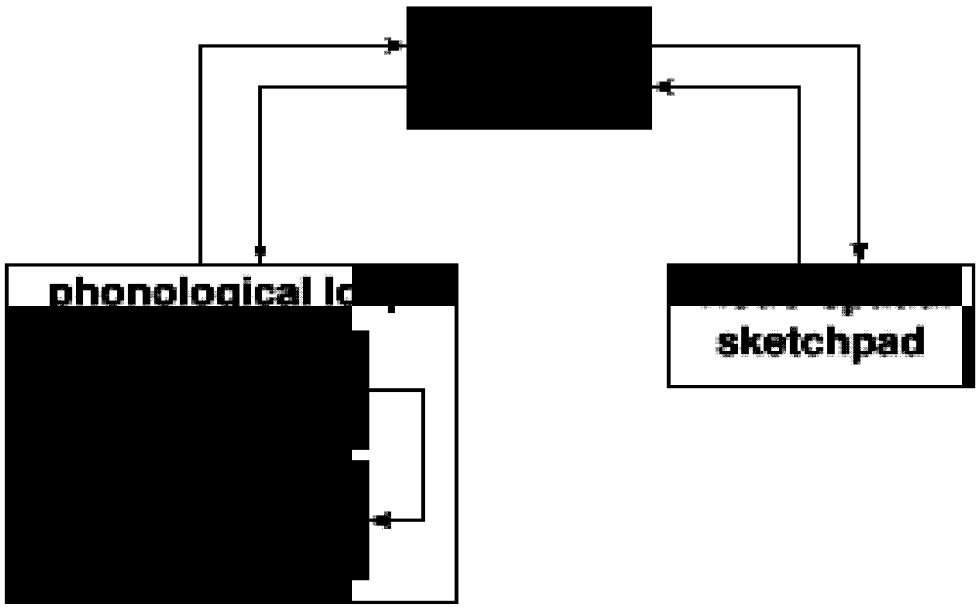

Figure 2.2: The working memory model components (Source: Baddeley and Hitch, 1974).

2.5 Studies Relating Hippocampus to Memory

Much of the current knowledge of memory has come from studying memory disorders. Loss of memory is known as amnesia. There are many sorts of amnesia, and by studying their different forms, it has become possible to observe apparent defects in individual sub-systems of the brain's memory systems, and thus hypothesize their function in the normally working brain. Other neurological disorders such as Alzheimer's disease can also affect memoryand cognition (Sutherland, 2001). Similarly, a lot of knowledge on memory stems from impact of direct lesions to parts of the brain even though most of the findings were accidental as seen in the case of Henry Gustav Molaison (HM) he had an intractable seizure for which he had a surgical removal of most of the medial temporal lobe. The unexpected outcome was severe antegrade and partial retrograde amnesia. H.M. was unable to form new episodic memories after the surgery and could not remember any events that occurred just before his surgery, but retained memories such as his childhood (Corkin, 2002). Similarly, a prospective study of retrograde amnesia in monkeys that had learned to discriminate 100 pairs of objects beginning 16, 12, 8, 4, and 2 weeks before the hippocampal formation was removed (Zola-Morgan and Squire, 1990). Memory was then assessed by presenting each of the 100 object pairs again for a single-choice trial. Normal monkeys exhibited forgetting; that is, they remembered recently learned objects better than objects learned many weeks earlier (Zola-Morgan and Squire, 1990). Monkeys with hippocampal damage were severely impaired at remembering recently learned objects, in addition, they remembered objects learned long ago as well as normal monkeys did and significantly better than they

remembered objects learned recently. These results show that the hippocampal formation is required for memory storage for only a limited period of time after learning.

As time passes, its role in memory diminishes, and a more permanent memory gradually develops independently of the hippocampal formation, probably inneocortex (Zola-Morgan and Squire, 1990). Nearly all investigators agree that any event which significantly alters the function of the hippocampus, either temporarily or permanently will cause amnesia in humans and rats. Well-studied examples include drug administration (Toumane and Derkin, 1993), electroconvulsive shock (Squire *et al*., 1981), brain trauma (Markowitsch *et al*.,1993), brain ischemia (Zola-Morgan *et al*., 1986), hypoxia (De Renzi and Lecchelli, 1993), encephalitis (Yoneda *et al*., 1992), surgical excision of brain tissue (Corkin, 1984), and even new learning(Keppel,1984). These events are associated with both anterograde and retrograde amnesia, i.e., performance is disrupted in tasks which depend on either post event learning or pre event learning. Similarly investigators have shown that cigarette smoke has damaging effect on the hippocampus (Olcay *et al*., 2007) with significant increase in the number of apoptotic bodies in hippocampus of rats exposed to cigarette smoke and other investigators that used neurological marker inferred that there is a decrease in cell number in the medial temporal lobe of primates exposed to cigarette smoke maintained at 4.3 ppm of carbon monoxide for six hours daily for five days in a week for six month (Slotkin *et al*., 2005).

2.6 Electrophysiology of Memory

Historically, Sir Hebb's coined a rule which states that synaptic transmission between two neurons will be strengthened if the two cells are simultaneously active and one cell repeatedly excites or causes the other cell to fire: it serve as a coincidence detector (Hebb, 1949).

This form of strengthening of synapses or synaptic plasticity is called long-term potentiation (LTP) and it clearly exists, as well as the reverse of LTP called long-term depression (LTD), a use-dependent decrease in synaptic strength. One implication of Hebb's rule suggests that improved coincidence detection between neurons will result in improved learning (Hebb, 1949). In neuroscience, LTP is a long-lasting enhancement in signal transmission between two neurons that results from stimulating them synchronously (Cooke and Bliss, 2006). It is the phenomena underlying synaptic plasticity, the ability of chemical synapses to change their strength. Since memories are thought to be encoded by modification of synaptic strength, (Bliss and Collingridge, 1993), hence LTP is widely considered one of the major cellular mechanisms that underlie learning and memory (Cooke and Bliss, 2006).

Similarly at the molecular level, long-term memory is widely believed to be expressed ultimately in the form of synaptic structural changes resulting from a single/ multiple molecular cascades. It is postulated that learning triggers a molecular cascade of events involving: a rapid, intense stimulation of CA1 neurons in the hippocampus which depolarizes them.

Binding of Glu and D-serine to their NMDA receptors opens them. Ca^{2+} ions flow into the cell through the NMDA receptors and bind to calmodulin. This activates calcium-calmodulin-dependent kinase II (CaMKII) (Sweath, 1999). CaMKII phosphorylates AMPA receptors making them more permeable to the inflow of Na^{+} ions and thus increasing the sensitivity of the cell to depolarization (Sweath,1999). In time CaMKII also increases the number of AMPA receptors at the synapse.

Increased gene expression (i.e: protein synthesis — perhaps of AMPA receptors) also occurs during the development of LTP (Frey *et al.*, 1996). Enlargement of the synaptic connections and perhaps the formation of additional synapses occur during the formation of LTP (Frey *et al.*, 1996).

Long term potentiation occurs in two phases: An early one (in the first hour or so) which involves increased sensitivity of the synapse without any new gene transcription or mRNA translation occurring and a late one which requires new gene transcription and mRNA translation and results in an increase in the number of AMPA receptors accompanied by an increase in the size of the synaptic connection.

These changes persist for many hours and even many days. However, increased AMPA receptor formation seems to require continuous enzymatic stimulation because (in rats, at least) interfering with the process erases late LTP (and memory) even a month later (Serrano *et al.*, 2005). This is yet more evidence that memories are acquired in two phases; early and late, and that the single cascade of molecular events may not explain long term memory. Late LTP may involve not only the addition of AMPA receptors to existing synapses but the formation of entirely new synapses (Whitlock *et al.*, 2006).

The NMDA receptor (NMDAR), is an ionotropic glutamate receptor and the predominant molecular device for controlling synaptic plasticity and memory function (Fei and Joe, 2009). NMDA (*N*-methyl *D*-aspartate) is the name of a selective agonist that binds to NMDA receptors but not to other glutamate receptors. Activation of NMDA receptors results in the opening of an ion channel that is nonselective to cations.

The NMDA receptor is distinct in two ways: First, it is both ligand-gated and voltage-dependent; second, it requires co-activation by two ligands - glutamate and glycine. This allows voltage-dependent flow of Na^+ and small amounts of Ca^{2+} ions into the cell and K^+ out of the cell (Paoletti and Neyton, 2007). The NMDA receptor is composed of an obligatory NR1 subunit and a selection of NR2 subunits (A–D). The NR2 subunit confers different channel properties on the receptor complex. One intriguing subunit difference is the longer excitatory postsynaptic potential (EPSP) observed in NR2B-containing receptors in comparison to NR2A-containing receptors. This longer EPSP suggests that NR2B-containing receptors have a longer time window for detecting pre- and postsynaptic activity resulting in increased coincidence detection and enhanced LTP when activity is less synchronized (Edward and Charles, 1999). Even the developmental regulation of NR2B supports the relationship between this subunit and increased LTP because the expression of NR2B is highest when the animal is young and decreases in adulthood, correlating well with the higher amount of LTP seen in younger animals (Edward and Charles, 1999). Interestingly, the calcium ion that enter the cell facilitates Ca^{2+}-stimulated adenylyl cyclase activity, which is essential for hippocampus-dependent long-term memory by eliciting the cascade of events that lead to protein synthesis and eventually synaptic structural changes as described earlier(Banke *et al*., 2000).

The foregoing is markedly illustrated in the work of Tang *et al*. (1999). In the study, transgenic mice, that over expressed the NR2B subunit in the forebrain under the control of the CaM kinase II (CaMKII) promoter were used. Using Northern blots, Western blots, and in situ hybridization, Tang *et al*., (1999) showed that NR2B mRNA levels are elevated in areas of the cortex and hippocampus with low expression in other areas of the brain.

To confirm that the increased expression had the desired effects on NMDA receptor conductance, they made whole-cell recordings from cultured hippocampal neurons at different time points. They found that the amplitude and duration of glutamate-evoked NMDA currents in the wild-type mouse dropped off sharply by 18 days in culture, but the amplitude and duration of NMDA currents in the transgenic lines remained high at 18 days. These investigators then examined LTP in these mice by doing extracellular field recordings in hippocampal slices from 4- to 6-months-old animals. LTP was substantially increased in the transgenic lines, and a lower frequency stimulus protocol (10 Hz tetanus) that does not induce LTP in control animals did induce LTP in the mutants, indicating improved coincidence detection by the NR2B-overexpressing neurons (Edward and Charles, 1999).

After demonstrating that their animal model did have increased LTP Tang *et al*. (1999) then tested for changes in learning and memory. They did this with a number of behavioral tests examining several types of learning such as novel-object recognition, associative fear learning, fear extinction and spatial learning. In these tasks, the transgenic animals clearly performed better than wild-type animals did by both learning faster and retaining memories longer (Edward and Charles, 1999). This study addresses a central debate in the field of neuroscience: Is LTP a cellular substrate of memory? This topic remains a matter of considerable controversy with strong proponents for either side. The strongest evidence

supporting a role for LTP in learning are experiments showing that a block of LTP is correlated with a decrease in learning and memory. Researchers have impaired LTP by both pharmacological agents and numerous gene knockouts and found deficits in two kinds of memory in two brain regions (hippocampal-dependent spatial memory and amygdala-dependent fear conditioning).

On the other hand, there are numerous exceptions to this correlation between LTP and memory with knockout mice that show diminished LTP with no deficit in spatial learning and mice with enhanced LTP but impaired performance in spatial memory tasks (Zamanillo *et al*., 1999).

Although the findings of Tang *et al.,*(1999). do not resolve the issue, they support the role of LTP in memory. Previous studies have analyzed assorted knockout mice in which the expected phenotype is impaired synaptic plasticity and behavioral performance. These experiments are questionable (and will always be questioned by critics) because of the possibility of developmental effects resulting in general and nonspecific animal defects (Edward and Charles, 1999).

However the next question is how many cascades or circle of activation of the entire molecular pathway will be sufficient to establish a synaptic change that will be sufficient to underline the phenomenon of long term memory? For the single cascade hypothesis that has guided experimental designs and conceptual thinking in the past decades, the following are strong issues against it.

First, hippocampus-mediated consolidation of long-term memories occurs over a time-scale of week(s) in rodents (Riedel *et al.*, 1999) and years in humans (Haist *et al.*, 2001).

Second, although protein synthesis inhibitors seem to produce long-term memory deficits (often tested within a day or days of training), spontaneous recovery or reminder-induced recovery of memory over a time course of a week or weeks has been reported in animals initially thought to be amnesic (Miller and Springer, 1974).

Third, synaptic structures in the adult brain are not stationary and synaptic receptors and proteins are turned-over regularly. A recent study using inducible-knockout techniques shows that synaptic NMDA receptors are degraded in approximately five days in the brain of freely behaving animals (Shimizu *et al*.,2000). Thus, it is not clear whether structural changes resulting from a single molecular cascade can be sustained in the presence of such dynamic turnover processes for long enough to complete cortical memory consolidation (Gayle *et al*., 2002).

Recent experiments using inducible gene- or protein-knockout techniques reveal that post-learning NMDA receptor and CaMKII reactivations are required for the systems-level consolidation of both hippocampal-dependent and hippocampal-independent memories (Huimin *et al*., 2006). Furthermore, the reactivations of the NMDA receptors are also necessary for the long-term storage of old memories in the neural circuits.

Therefore, the NMDA receptor reactivation-mediated synaptic reentry reinforcement (SRR) process may represent the unifying cellular mechanism in linking the consolidation and storage of long-term memories from the molecular level to the systems-level. The central idea of the SRR hypothesis is that reactivation of neural ensembles in the hippocampus during the consolidation period results in multiple rounds of NMDA-receptor-dependent synaptic reinforcement of the hippocampal memory traces created during initial learning.

Such a process could counteract the turnover of synaptic receptors, making memory traces stronger and more stable, extending their durability and robustness within the hippocampus (Huimin *et al.,* 2006).

In addition, such reactivation and reinforcement processes permit the hippocampus to act as a 'coincidence regenerator', providing coordinated input that drives the coherent reactivation of cortical neurons, resulting in the progressive strengthening of cortical memory traces through reactivation of cortical NMDA receptors (Huimin *et al.,* 2006).

2.6.1 SRR And Hippocampal Memory Consolidation

Shimizu *et al.* (2000) showed that the formation of long-lasting memories was severely disrupted if the CA1-hippocampal NMDA receptor was knocked out selectively during the initial post-training week(s) (Shimizu *et al.,* 2000*)*. By contrast, inducible knockout of the CA1-hippocampal NMDA receptor during the fourth week post-training had no effect on the retention or retrieval of one-month-old hippocampal memories. These results are consistent with lesion studies showing that the hippocampus becomes dispensable once memories have been sufficiently consolidated in the cortex (Anagnostaras *et al.,* 1999). Numerous questions arise regarding SRR, e.g: When and how does SRR occur? Is there a role for sleep? What might trigger the SRR process and when does SRR take place? It is conceivable that one triggering mechanism could be conscious recall. This is consistent with general experience: if a particular event is recalled more frequently, it will be remembered more efficiently and for longer. Another triggering mechanism could be the subconscious reactivation of the hippocampus and/or cortex, which could be achieved during sleep. An increasing amount of evidence suggests a role for sleep in memory consolidation (Maquet, 2001). Moreover,

learning-induced correlations in the firing of hippocampal place cells have been reported to reappear during sleep (Louie and Wilson, 2001).

Such coordinated reactivation of these neurons suggests the existence of a natural condition in which the NMDA receptor might be reactivated, thus reinforcing the synaptic connections between them.

2.7 Memory Tasks For Rats

There are numerous tasks that are used to demonstrate the various types of memories in mammals. The tasks include the radial arm maze, inhibitory avoidance, Morris water maze. Morris water maze training is specific for exerting the function of spatial memory and its brief description is:

2.7.1 Morris Water Maze Task

Morris water maze is one of the most widely used tasks in behavioral neuroscience for studying the psychological processes and neural mechanisms of spatial learning and memory. The basic task is very simple. Animals, usually rats or mice, are placed in a large circular pool of water and required to escape from water onto a hidden platform whose location can normally be identified only using spatial memory. There are no local cues indicating where the platform is located. Conceptually, the task derives its foundation from place cells, which are neurons in the hippocampus which identify or represent points in space in an environment (O'Keefe, 1976). It was developed by Richard Morris at the University of St Andrews in Scotland and first described in two publications in the early 1980s (Morris *et al*., 1982). Place navigation in the water maze is now often used as a general assay of cognitive function (Brandeis *et al*., 1989), for example for testing the

impact of various disturbances of the nervous system e.g. animal models of stroke (Nunn *etal*., 1994), aging (Gallagher and Rapp, 1997), neurodegenerative disease (Hsiao *et al*., 1996), or the potential impact of novel therapeutic drugs (D'Hooge and De Deyn, 2001).

The task has also inspired computational neuroscientists and roboticists interested in navigation (Krichmar *et al*., 2005).

The apparatus consists of a large circular pool, generally 1.5 to 2 metres in diameter, containing water at around 25°C made opaque by adding milk or another substance (e.g. latex liquid) that helps to hide the submerged platform. The water in the pool is filled and drained daily, via an automated filling and draining system. This choice of water temperature at around 13°C below body temperature is sufficiently stressful to motivate the animals to escape, but not so stressful as to inhibit learning. There is a mild stress reaction on day 1 of training (indicated by elevated corticosterone), but this habituates over days (Sandi *et al*., 1997). If the pool temperature drops to 19°C, performance improves, but when temperature drops to 12°C, it gets worse – reflecting the inverse U-shaped function relating stress to cognitive function. The top surface of the hidden platform, usually about 10-15 cm in diameter and thus between $1/50^{th}$ and $1/100^{th}$ of the surface area of the pool, is 1.5 cm below the water surface. The pool itself should be located in laboratory room with distinctive 2- and 3-D distal cues that aid orientation, with 3-D cues being particularly helpful. Alternatively, the pool may be surrounded with hanging curtains that occlude fixed room cues, enabling moveable cues to be hung inside that can be rotated relative to the room when this degree of precise experimental control is required.

Again, it is best if these are 3D rather than flat surfaces. The use of a cue-controlled environment has proved particularly helpful in studies of pattern completion (Nakazawa *et al*., 2002). Several protocols or approaches can be utilized in assessing the neuro-behaviour of animals including the following among others.

Reference memory protocols (RMP) are widely used in which the platform is in a fixed location relative to the room cues across days.

The animals are placed into the water facing the sidewalls of the pool, at different start positions across trials, and they quickly learn to swim to the correct location with decreasing escape latencies and more direct swim paths. The tracking system measures the gradually declining escape latency across trials, and parameters such as path-length, swim-speed, directionality in relation to platform location, and so on. Observation of the animals reveals that, having climbed onto the escape platform, they often rear up and look around, as if trying to identify their location in space. Rearing habituates over trials, but then dishabituates if the hidden platform is moved to a new location (Sutherland and Dyck, 1984).Probe trials to test memory (PTTM) during or after training, the experimenter conducts a probe trial in which the escape platform is removed from the pool and the animal allowed to swim for 60 sec. Typically, a well-trained rat will swim to the target quadrant of the pool and repeatedly across the former location of the platform until starting to search elsewhere. This spatial bias, measured in various ways, constitutes evidence for spatial memory. Rats with lesions of the hippocampus and dentate gyrus, subiculum, or combined lesions, do poorly in post-training probe tests (Sutherland *et al*., 1983). However, if rats with hippocampal lesions are given overtraining (typically consisting of a large number of trials over many days), their performance in probe tests can be quite good (Morris *et al*., 1990).

Even rats with hippocampal lesions can show quite localized searching particularly if the most septal pole of the longitudinal axis of the hippocampus is spared (Moser *et al*., 1993).

The durations of training necessary to depict short term memory and long term memory is exemplified in the work of Barros *et al*. (2005) with memory tested after 90 min of training for short-term memory while as memory testing after 24h of training for long-term memory training. Memory could be affected or distorted by trauma / surgical ablation and numerous agents like nicotine, carbon monoxide, cortisol and cigarette smoke (Sutherland *et al*., 1983).

2.8 Cigarette/ Cigarette Smoke

Cigarette smoking is the most widely practiced and researched modality of usage of tobacco (Virginia, 2001) other modalities of using tobacco include: Consumption either by chewing , snuffing, smoking or dopping, usage as organic pesticides and as medicine.Tobacco smoke consists of 10% solid particles and 90% gasses, up-to 4000 constituents have been identified so far 30-60 of which are known carcinogen (Rudnick, 1998). The fertilizer (of the appetite variety) used during cultivation of the tobacco plants starves the plant of nitrogen in order to enhance the flavor of the cigarette, however the appetite fertilizer is rich in radioactive substances like Polonium 210, Lead 210, Radium 226, Potassium 40 and Radium 228 which are potent carcinogens (Wingand, 2006). During curing, processes of conversion of starch to a sugar can glycates proteins during smoking. Oxidation of these glycated proteins during smoking lead to the formation of advanced glycation end products (AGES) which contribute to atherosclerosis and cancer (Cerami *et al*., 1997).

2.8.1. Carbon Monoxide

Carbon monoxide (CO) is a colourless, odourless and tasteless gas. The largest source of carbon monoxide is natural in origin, due to photochemical reactions which generate about 5 x 10^{12} kilograms per year (Weinstock and Niki, 1972). Other natural sources of CO include volcanoes, forest fires, and other forms of combustion including side stream smoke which contains 6.8 times more carbon monoxide than mainstream smoke (Williams *et al.,* 1984). Carbon monoxide occurs in various natural and artificial environments at different concentrations: Table 2.1 shows the concentration of carbon monoxide in the vicinity of different sources in the Environment

Table 2.1: Showing the concentration of carbon monoxide in the vicinity of different sources in the Environment

Concentration	Source
0.1 ppm	Natural atmosphere level (MOPITT) (CMBEEP, 1977)
0.5 to 5 ppm	Average level in homes (Green, 2008)
5 to 15 ppm	Near properly adjusted gas stoves in home (Gosink, 1983)
100-200 ppm	Exhaust of automobiles in Mexico area (Singer, 1975) .
5,000 ppm	Exhaust from a home wood fire (Gosink, 1983).
7,000 ppm	Undiluted warm car exhaust without a catalytic converter (Gosink, 1983).

Sources : (1)Committee on Medical and Biological Effects of Environmental Pollutants (1977). *Carbon Monoxide*. Washington, D.C.: National Academy of Sciences. pp. 29.(2) Gosink, 1983. (3) Singer, 1975. (4) Green, 2008.

Carbon monoxide is often referred to as the "silent killer" because it is virtually undetectable without using a detection technology and detection of carbon monoxide in the atmosphere is achieved by CO detector. The detectors are designed to measure CO levels over time and sound an alarm before dangerous levels of CO accumulate in an environment, giving people adequate warning to safely ventilate the area or evacuate. Some system-connected detectors also alert a monitoring service that can dispatch emergency services if necessary (US.CPSC Document number 5010).

Interestingly, carbon monoxide, previously thought to be a toxic gas and biological waste product is now being considered a likely candidate in the new class of gaseous neural messengers (Kolata, 1993).In animals, the predominant source of carbon monoxide generation is heme degradation. Hemeoxygenase (HO) is the rate limiting enzyme responsible for the catabolism of heme and subsequent production of carbon monoxide and bilirubin. There are two types of HO; type 1 commonly seen in the liver and spleen and type 2 commonly seen in the brain and testis (Stefan *et al*., 2006).

The toxicity of carbon monoxide is a function of its ability to form carboxyhaemoglobin (COHB), a stable chemical complex with haemoglobin. This effectively serves to remove oxygen-carrying haemoglobin from the circulating blood and to vital tissues. Similarly, carbon monoxide binds to mitochondrial cytochromes of respiring cells *in vitro* only when either the CO concentration was extraordinarily high, or oxygen tension was extremely low, such that the CO/O2 ratio exceeded 12:1 (Coburn and Forman, 1987). Following CO exposure and removal to fresh air, CO diffuses out from the cells, and mitochondrial function is restored. This process could be enhanced by inspiration of hyperbaric oxygen (Brown and Pinantadosi, 1992).

Studies in mice indicate that high CO concentrations inhibit synthesis of high- energy phosphate during exposure to 5000ppm CO for 15 mins and these changes do not persists following removal to fresh air (Matsuoka *et al*., 1993). It is well known that severe hypoxia due to acute carbon monoxide poisoning may cause both reversible neurological deficits and severe, often delayed, neurological damage (ACGIH, 1991). However, the effects of carbon monoxide on cognitive performance have generally been equivocal at COHB levels of 5-20% (Laties and Merigan, 1979). Several other studies in humans have produced numerous data regarding the level of COHB in the blood attributable to certain symptoms. COHB concentrations in the blood of about 2% or more of hemoglobin have been associated with angina pain in people with cardiovascular disease and can result in cardiac ischemia and diminished blood flow to the heart (Smith *et al*., 1997). A mild headache at 10-20% level of COHB, while throbbing headache, irritability, emotional instability impaired judgement, defective memory and rapid fatigue at COHB LEVEL of 20 – 30%. Similarly there is severe headache, weakness, nausea, vomiting, dizziness, dimness of vision, confusion as the common symptoms that are seen when the level of COHB is 30-40% (Gosselin *et al*., 1984).

2.8.1.1. Carbon Monoxide and Long Term Potentiation

A landmark experiment in which hippocampal slices from rats exposed to 150 PPM of CO resulting in blood levels of carboxy-hemoglobin comparable to those found in human cigarette smokers during development exhibited an impaired ability to maintain long term potentiation (LTP) over time, in association with a modest, but significant, reduction of post titanic potentiation (PTP) and in the absence of any observable difference in baseline synaptic transmission between control and CO-slices.

Indeed, input /output (I/O) function, presynaptic volley, prê-tetanus paired –pulse facilitation (PPF), and percentage of slices showing PTP were not affected by the gestational exposure to CO (Giampaolo *et al*., 2000). These results provided the first evidence that altered cellular mechanisms in rat hippocampus might underlie learning and memory deficits induced by prenatal exposure to CO at a concentration (150 ppm) approaching that experienced by offspring of mothers who smoke during pregnancy (Giampaolo *et al*., 2000). Indeed, previous studies have shown that prenatal exposure to 150 ppm of CO impairs cognitive functions in rat offspring, without affecting non associative or motivational parameters (Di Giovanni *et al*., 1993). LTP in the CA1 region of the hippocampus reflects cellular physiological changes critical to the processes of learning and to the intermediate stages of memory consolidation and retention (Nicoll and Malenka, 1995). Different mechanisms regulate early and late phases of LTP in CA3-CA1 synapses of the hippocampus. Thus, whereas LTP induction requires Ca^{2+} influx via *N*-methyl-d-aspartate (NMDA)-ionotropic receptors, expression and maintenance appears to be supported by various factors located on both pre- and postsynaptic sites (Bliss and Collingridge, 1993). These factors include an enhanced glutamate release sustained by the production of retrograde messengers, such as NO and CO itself (Medina and Izquierdo, 1995). Learning and memory deficits induced in rat offspring by prenatal exposure to CO could stem, at least in part, from the disruption of the processes required for long-term synaptic storage, as reflected by the suppression of LTP maintenance, possibly via a negative action on the heme-oxygenase2(HO) and nitric oxide synthetase (NOS) enzymes.

2.8.2 Nicotine

Nicotine is an alkaloid found in the nightshade family of plants (Solanaceae) that constitutes approximately 0.6–3.0% of dry weight of tobacco,(Barbara, 1999)with biosynthesis taking place in theroots and accumulation occurring in the leaves. It functions as an anti-herbivore chemical with particular specificity to insects; therefore nicotine was widely used as an insecticide in the past, (Thomas and Laura, 2010) and nicotine poisoning was once relatively common due to its use in insecticides in the 1920s and 1930s, but rare these days (Gosselin *et al.,* 1984). Dosages of nicotine obtained through tobacco smoking are far too low to cause acute poisoning, since the average lethal dose of nicotine is 30-60 milligrams (mg) which is far more than the concentration obtainable by cigarette smoking. However it is worthy of note that the nicotine content in cigarettes has slowly increased over the years, and one study found that there was an average increase of 1.6% per year between the years of 1998 and 2005 and this was found for all major market categories of cigarettes (Connolly *et al*., 2007), yet it is still far from the lethal dose of 30- 60mg for an adult human.

Nicotine is metabolized in the liver by cytochrome P450 enzymes (mostly CYP2A6, and also by CYP2B6). A major metabolite is cotinine (half life of 18 - 20 hours) (Oneil, 2003) other primary metabolites include nicotine N'-oxide,nor -nicotine, nicotine isomethonium ion, 2-hydroxynicotine and nicotine glucuronide. Glucuronidation and oxidative metabolism of nicotine to cotinine are both inhibited by menthol. Nicotine can be quantified in blood, plasma, or urine to confirm a diagnosis of poisoning or to facilitate a medico-legal death investigation (Baselt, 2008). Urinary or salivary cotinine concentrations are frequently measured for the purposes of pre-employment and health insurance medical screening programs.

Careful interpretation of results is important, since passive exposure to cigarette smoke can result in significant accumulation of nicotine, followed by the appearance of its metabolites in various body fluids (Benowitz *et al.,* 2009). It is ironic that except in the case of tobacco products, which are expressly manufactured for self-administration by humans at great loss of life, products containing nicotine are highly regulated (NHMRC, 1991; 1992).

The immediate physiological effects of nicotine include increased heart rate and blood pressure, constriction of cutaneous blood vessels, and muscular, hormonal and metabolic effects (U.S.DHH, 1988). With prolonged exposure, it may contribute, in combination with carbon monoxide, to increased platelet stickiness and aggregation and damage to the lining of the blood vessels, suggesting a potential role in causing coronary disease (NHMRC, 1991; 1992). It is also implicated in the causation of reproductive, gastrointestinal disorders and it enables the formation of tobacco-specific nitrosamines, which are potent carcinogens (U.S.DHH, 1988). Products containing nicotine for therapeutic (drug) use are categorised as Schedule 3 and 4 poisons (NHMRC, 1991; 1992).

2.8.2.1 Nicotine and Cognition

Nicotine is an important acute-acting pharmacological agent in tobacco smoke, and is the addictive ingredient in tobacco that drives people to dependence but it has also been shown to improve cognitive function in humans and laboratory animals (Levin *et al*., 2005; Mansvelder *et al*., 2006).In smokers and patients suffering from a variety of neuropsychiatric disorders, nicotinic agonists act beneficially on several aspects of cognition, including working memory attention, learning, and memory.

In fact, nicotinic treatments are being developed as therapy for cognitive dysfunction in disorders such as Alzheimer's disease, Parkinson's disease, schizophrenia and attention deficit hyperactive disorder (ADHD) (Levin *et al*., 2005). In contrast, in normal nonsmokers, nicotine tends to have deleterious effects on cognitive performance (Newhouse *et al*., 2004b). Although it is likely that many brain areas contribute to the nicotinic effects on cognition, based on animal studies nicotinic acetylcholine receptors (nAChRs) in the prefrontal cortex (PFC) mediate the effects on attention and working memory performance (Granon *et al*., 1995). The rodent medial PFC is considered to be functionally homologous to the primate dorso-lateral PFC and has been shown to be involved in attention and working memory (Dalley *et al*., 2004). And other studies have found that α7 and α4β2 nicotinic receptors in the hippocampus are critical for nicotinic involvement in cognitive function. Clinical and experimental animal studies provide mutually supporting information for the development of novel nicotinic therapies for cognitive dysfunction.

2.8.2.2 Nicotine and Hippocampus

Animal studies show that nicotine damages the developing brain by altering the formation, survival and differentiation of brain cells, eliciting deficits in structure, synaptic function and behavioural performance (Walker *et al*., 1999). Several mechanisms are involved in the affectation of the brain by nicotine culminating in reduced cell number (Slotkin, 1992, 1998) and aberrant cell proportions (Roy *et al*., 1998). Adolescent nicotine treatment evoked decreases in total cell number as assessed by DNA measurements in cerebral cortex, midbrain and hippocampus (Trauth *et al*., 2000).

Although the effects were smaller in magnitude (5–10%) than had been seen with the fetal exposure model, it should be noted that these are very tightly controlled parameters: except for a few circumscribed regions, brain cell number does not change after the closure of neural mitosis in the early postnatal period (Slotkin, 2002). Additional measures that might reinforce the concept of changes in cell number or size using the membrane protein concentration in the hippocampus showed a robust, progressive increase in the concentration of membrane proteins in the hippocampus of adolescent female rats after exposure to nicotine, associated with a surge in *p53* expression (Trauth *et al*., 2000). In contrast, studies with adult rats indicate short-term cognitive enhancement by nicotine (Mirza and Stolerman, 1998) stimulation of nicotinic cholinergic receptors in mature cells can actually decrease the cell death elicited by injurious treatments (Zoli *et al*., 1999), potentially by induction of neurotrophic factors (Maggio *et al*., 1997, 1998). Clearly then, the developmental stage at which nicotine exposure occurs is critical in determining the outcome.

However, other studies show that nicotine act through their ability to generate free radicals which generates an oxidative stress (Gitto *et al*., 2002, Gupta, 2004). Similarly nicotine shows an hypoxic-ischemic effect (Mc Farlands, 1991),which is most marked when the serum concentration of nicotine is elevated and could contribute to the damage of neural structures.

2.8.3 Hydrogen Sulphide

Information on the neurotoxic effects of exposures to high concentrations of hydrogen sulfide in humans comes primarily from case reports and in most instances, exposure concentrations were either unknown or estimated: Single exposure to high concentrations of hydrogen sulfide can cause nausea, headaches, delirium, disturbed equilibrium, poor

memory, neuro-behavioural changes, olfactory paralysis, loss of consciousness, tremors, and convulsions (Zenz *et al*., 1994). Fatigue, poor memory, dizziness, and irritability have been observed in workers chronically exposed to hydrogen sulfide (Beauchamp *et al*., 1984). Three men accidentally exposed to hydrogen sulfide in excess of 350 mg/m3 after a few minutes became unconscious (McDonald & McIntosh, 1951). Loss of consciousness has also been reported with single exposures to estimated concentrations of 700-2800 mg hydrogen sulfide/m3 (Deng and Chang, 1987). Other described neurological effects in case reports included disturbed equilibrium, nausea, headache, poor memory, insomnia, irritability, delirium, vertigo, unusual sweating, neuropsychological symptoms, convulsions and tremors (Arnold *et al*., 1985). The neurological effects following single inhalation exposures to high concentrations of hydrogen sulfide may be permanent or persistent. A 5- to 10-year follow-up re-examination of several workers who become (sic)unconscious after exposure to unspecified concentrations of hydrogen sulfide revealed permanent neurological symptoms (Tvedt *et al*., 1991a, 1991b), including vision and memory impairment, rigid movements, reduced motor function, slight tremor, ataxia, psychosis, abnormal learning, retention, and motor function, and slight cerebral atrophy.

Similarly, Neurological effects resulting from long-term exposure to hydrogen sulfide in the shale industry have also been reported (Ahlborg, 1951). Symptoms observed in workers exposed to daily concentrations of hydrogen sulfide that often exceeded 28 mg/m^3 included fatigue, loss of appetite, headache, irritability, poor memory, and dizziness. The frequency of fatigue increased with length of employment and the degree of hydrogen sulfide exposure.

In rodents, morphological examination of cerebellar Purkinje cells from Sprague-Dawley rat pups exposed to 28.70 mg hydrogen sulfide/m3 for 7 h/day from gestation day 5 through postpartum day 21 showed severe alterations in the architecture and growth characteristic of the Purkinje cell dendritic fields compared with controls (Hannah and Roth, 1991). These findings suggest that developing neurons exposed to low levels of hydrogen sulfide are at risk of developing severe deficits (WHO, 2003).

2.8.4 Tar

"Tar" is defined as the nicotine-free, dry, particulate mass of tobacco smoke. The particulate fraction of cigarette smoke contains many harmful carcinogenic constituents, including metals. Polynuclear aromatic hydrocarbon (PAH), dioxins, and some non-volatile nitrosamines. The nature of the chemical components in tar and their toxicity vary widely across tobacco from various sources. Therefore, measurement of Tar, is only a crude measure of the relative toxic potential of tobacco combustion products (U.S.DHH, 1988).

2.8.5 Polynuclear Aromatic Hydrocarbon (PAH)

Polynuclear aromatic hydrocarbon (PAH) Benzo(a) pyrene (BaP) is the most commonly studied and one of the most toxicologically potent of these compounds. Benzopyrenes are problematic because they intercalate into DNA, interfering with transcription (Denissenko *et al*., 1996). It is considered a pollutant and carcinogen. There are evidences to link benzopyrene to the formation of lung cancer (Denissenko *et al*., 1996).

2.8.6 Chlorinated Dioxins and Furans

Chlorinated dioxins and furans collectively referred to as "dioxins" are ubiquitous environmental contaminants formed through the reaction of organic matter and chlorine,

often under conditions of combustion (Wikstrom *et al*., 2004). A report on dioxin levels of Swedish cigarettes showed dioxin levels of 1490 pg/20 cigarettes mainstream smoke (Lofroth and Zebuhr, 1992).

Similarly, metals have been detected in tobacco smoke, including nickel, arsenic, cadmium (WHO 1985); chromium and lead (U.S DHH, 1988). High level of cadmium and arsenic may be related to the presence of cadmium in phosphate fertilizers and arsenic in arsenic pesticides (WHO, 1985).

2.8.7 Cigarette Smoke in The Environment

The concentration of the various Environmental Tobacco Smoke (ETS) constituents in an indoor space depends on the number of smokers, the volume of the space, the ventilation rate. It also depends on the effectiveness of the air distribution, the rate of removal of ETS from the indoor air by air cleaners, deposition of particles onto surfaces and surface adsorption and re-emission of gaseous components. Because ETS is a complex mixture, measurements of single components are of varying specificity and none alone is considered to indicate the potential toxicity of ETS at a particular concentration. Therefore, measurements of multiple surrogates have been used as indicators of the concentration of the mixture for research and public health purposes (ASHRAE, 2008). These measures include respirable suspended particles (RSP), nicotine, benzene, solanesol, 3-ethenyl pyridine (3-EP) and carbon monoxide. Such measurements have demonstrated contamination of indoor air wherever smoking takes place. Biomarkers of ETS exposure, i.e., indicators in biological materials such as nicotine in saliva and blood, have also been measured; measurable concentrations of these biomarkers (e.g. cotinine) have been found in the bodies of exposed nonsmokers, indicating uptake of ETS (Rudnick, 1998; ASHAREA, 2008.).

Interestingly, for inhalational studies, there is a standard method for collection of cigarette smoke that have been developed by the Federal Trade Commission (FTC). The FTC method of assessment of concentration of cigarette smoke constituent takes a typical smoking pattern to consist of one 35 cm^3"puff" of 2 seconds duration, once per minute. And demarcates the smoke into Mainstream (MS) smoke: is the smoke which is directly inhaled by the smoker and side stream (SS) or secondhand smoke: is the smoke which is released to the environment from the burning cigarette. An abridged version of the report of The Laboratory of the Government chemist (LGC) for the FTC is gazette yearly. An abridged version of the gazette report for the year 1999 is presented in table 2.2.

Table 2.2: Table of tar, nicotine and carbon monoxide for UK brands (abridged)

NFDPM YIELD mg/cig	BRAND NAME (SURVEY 43 JANUARY TO DECEMBER 1999)	NICOTINE YIELD mg/cig	CO YIELD mg/cig
1	MAYFAIR ULTRA LIGHTS	0.1	1
1	SILK CUT 100S ULTRA	0.1	1
1	SILK CUT SUPER LOW KING SIZE	0.1	1
1	SILK CUT ULTRA	0.1	1
2	LAMBERT & BUTLER ULTRA	0.2	2

Source: A survey of 2OO UK brands (Jan-Dec 1999) for tar, nicotine and carbon monoxide yields in cigarette smoke TABLE As determined by LGC Limited and Arista Laboratories Europe (*) for 184 brands available during the period. The LGC gazette for the year 1999.

A number of investigators have also attempted to measure the levels of some of the substances in cigarette smoke encountered in experimentally controlled situations. The type and amount of tobacco product burned, size of the room, amount and type of ventilation or filtration, duration of the smoking, as well as background atmospheric contamination, have all been shown to influence the measured concentrations and absorption by the non-smoker. A number of substances have been the subject of particular investigative attention including carbon monoxide and nicotine. Several investigators have tried to determine the amount of carbon monoxide absorbed in involuntary smoking situations by measuring changes in carboxy-hemoglobin levels in non-smokers exposed to cigarette smoke-filled environments. Anderson and Dalhamn (1973) found no change in the COHb levels of non-smokers in a well-ventilated room where the CO level was 4.5 ppm. When Harke (1970) studied nonsmokers under similar conditions (good ventilation and less than 5ppm CO),he found an increase in COHb level from 1.1 to 1.6 percent; without Ventilation the CO levels rose to 30ppm and the COHb level increased from 9 to 2.1 percent in 2 hours. Russell et al.,(1973) found that COHb levels increased from 1.6 to 2.6 percent in nonsmokers present in a smoke-polluted room where the CO level was measured at 38 ppm; however, they cautioned that nearly all persons in the room felt that the conditions were worse than those experienced in most social situations. Aronow (1980) exposed 10 patients with coronary artery disease to the smoke from 15 cigarette smoked by 3 volunteers over two(2) hours in a 30.8m^3 room. He reported that the COHb levels increased in the nonsmokers from a baseline of 1.26 percent to 1.77 percent when the room was ventilated at 11.4 air changes per hour and from 1.36 percent to 2.28 percent when the Ventilation was turned off.

Stewart *et al*. (1970) measured COHb levels in a group of non-smoking blood donors from several cities and found that 45 percent exceeded the Clean Air Acts Quality Standard of 1.5 percent, with the 96 percent range as high as 3.7 percent for individual cities. These levels represent the total body burden of CO for the non-smoker due to endogenous production as well as to all forms of environmental exposure (industrial and automobile as well as smoking). They are also the levels from which any increase would occur when the nonsmoker encounters an environment in which smoking has raised the ambient CO levels. Table 2.3 displays some of the values obtained for carbon-monoxide concentration in various experimental settings.

Table 2.3.-Measurement of constituents of tobacco smoke in experiments

Reference, location and dimension	Ventilation	Amount of tobacco burned	Level of carbomonoxide
De rouane and verduyn House 60m3	Closed	3 cig., in 34min	7.5pp
Harke ROOM 57M3	NONE 7.2 air changes / hr 8.4 air changes /hr None 7.2 air changes /hr	42 CIG IN 18MIN ‘’ “ 9 CIG IN 35MIN ‘’	50MM mil/mg/m3 10ppm CO <10 60PP 1Mmg/m3 20WM .42mg/m3
Dublin Conference room 138m3	12.0 air changes per hour	2 cig.	32.5pp

Source: United States. Public Health Service Office on Smoking and Health (1979). Smoking and Health: A Report of the Surgeon General" (pages 11-8 through 11-32) *Editor:* Pinney, John M. DHEW Publication No. (PHS) 79-50066

2.8.7.1 Cigarette smoke exposure and the hippocampus

Exposure of a group of rabbit to cigarette smoke and another group of rabbits to Caffeic acid phenethyl ester(CAPE), just before cigarette smoke exposure respectively for the same length of time, linked cigarette smoke directly to the structural and apoptotic changes in hippocampus (Olcay *et al*., 2007). Since the CA1, CA2, CA3 and dentate gyrus areas showed an increase in apoptotic bodies in the group of rabbits that were exposed to the cigarette smoke while as the rabbits in the control or no smoke exposure group and those in the CAPE and smoke exposure showed little or no changes . Hence the CAPE compound confers a neuro-protective effect on the neurons of the hippocampus against the changes induced by the cigarette smoke (Olcay *et al*., 2007).

Another experiment in which neuro-chemical markers were used to map out the morphological characteristics of various brain region following exposure to environmental tobacco smoke is of importance. Environmental tobacco smoke (ETS) was delivered using two inhalation chambers, each with an air capacity of 3.5 m^3, with each housing two monkeys. Aged and diluted side stream smoke was used as a surrogate for ETS. Standardized 1R4F research cigarettes were smoked simultaneously with a single puff volume of 35mL per cigarette and duration of 2 sec, once per minute. Airflow through the system was set for 30 changes per hour and samples were collected daily to determine the concentrations of total suspended particulates, nicotine (average, 162 $\mu g/m^3$) and carbon monoxide (average, 4.3 ppm). These concentrations represent the high end of field measurements reported for household ETS but are within the range of what a child would experience if the caretaker is a smoker (Jenkins *et al*., 2000; USEPA, 1992).

The exposure set up described is called a dynamic exposure chamber. Exposure to ETS occurred for 6 hr/day, 5 days/week, beginning at about GD50; pregnant animals in the control and postnatal exposure groups received filtered air in the same apparatus on the same schedule. All dams were allowed to give birth spontaneously. ETS or filtered air exposures were continued through 13 months postnatal, with the chamber containing both the mother and infant until removal of the mother at weaning of 5 months of age. The group with ETS exposure limited to the postnatal period was switched from filtered air to ETS at 6 months of age and continued through 13 months. At 13 months, the offspring were anesthetized with ketamine (10 mg/kg intramuscular) and euthanized with pentobarbital (80 mg/kg intravenous). The heart was dissected and brain samples were taken from the three regions of the cerebral cortex (frontal, temporal, and occipital cortex) as well as the midbrain, using anatomical landmarks to ensure sampling of the same area from each monkey. DNA was assessed with a modified (Trauth *et al*., 2000) fluorescent dye-binding method (Labarca and Piagen, 1980). Samples were then read in a spectrofluorometer using an excitation wavelength of 356 nm and an emission wavelength of 458 nm and were quantitated using standards of purified DNA. The total concentration of tissue proteins was assayed from the original homogenate spectrophotometrically with bicinchoninic acid (Smith *et al*., 1985); in addition, assessment of the concentration of membrane proteins from the membrane preparations used for radio-ligand binding was conducted in the study, for calculation of the ratio of membrane/total protein, the membrane protein value was averaged across the different membrane preparations. The DNA concentration, an index of cell packing density, showed regionally selective changes elicited by ETS exposure.

Although values were unaffected in the frontal cortex, both the occipital cortex and midbrain displayed significant increases after either continuous ETS exposure or ETS exposure restricted to the postnatal 6-13 month period (Slotkin *et al*., 2006). In contrast, values tended to be reduced in the temporal cortex, achieving statistical significance with the postnatal exposure group. Both indices of cell size also displayed ETS-induced differences. For the total protein/DNA ratio, the values were reciprocally related to the change in DNA concentration (Slotkin *et al*., 2006). Accordingly, reductions were seen in the occipital cortex and midbrain, whereas an increase was obtained in the temporal cortex (Slotkin *et al*., 2006).The membrane/total protein ratio showed overall increases that were not regionally selective but that were statistically significant both for continuous ETS exposure and for the group receiving only postnatal exposure. In Conclusion, perinatal or postnatal ETS exposure elicited two characteristic patterns of neural cellular effects, both of which resemble earlier findings for effects of prenatal nicotine exposure in rodents (Slotkin, 1998, 2004). In the occipital cortex and midbrain, there were smaller cells (reduced total protein/DNA ratio) and a corresponding increase in cell packing density (DNA concentration), features that are likely to reflect neuronal damage and "reactive gliosis," that is, replacement with smaller, glial cells (Roy *et al*., 1998, 2002). In contrast, in the temporal cortex, there were reductions in the total number of cells (reduced DNA) with hypertrophy of the remaining cells (increased total protein/DNA ratio), changes indicative of cell loss with perikaryal swelling (Roy *et al*., 2005). Superimposed on these two patterns, is an overall increase in the membrane/total protein ratio, which is compatible either with smaller cells (higher surface-to-volume ratio) or with increased neuritic sprouting.

CHAPTER THREE

3.0 Materials and Method

3.1 Materials

3.1.1 Rats

30 experiments naïve Wistar rats were obtained from the animal house of the Biological sciences Department of Bayero University Kano and transported to the animal house in the Department of Human Anatomy, Ahmadu Bello University Zaria. The animals were separated into females and males and also into experimental and control groups of 5 animals per cage respectively. The cages were made of plastic sides and an iron mesh lined with wood dust as the floor. The animals were allowed to acclimatize for two (2) weeks in their new environment. The animals were allowed 12 hour light and dark cycle and supplied with feeds from the sanders feed company ad libitum. Water was served by using plastic feeding bottles tactically placed in between the wire mesh roofing. Animals were weighed on daily basis and were in the weight range of 150- 250 g.

3.1.2 Carbon monoxide meter

The carbon monoxide meter CM100 from Amprobe was used. The meter has the following technical specifications; carbon monoxide range is 0-999ppm, a resolution of 1ppm and accuracy of +/- 20% at 0- 100ppm; +/- 15% at 100 -500ppm.The meter has a digital display screen and a backlight for operation in dark areas. The carbon monoxide meter is shown in plate 3.1

3.1.3 Nicotine screening strip

Nicotine screening strip comprising of a well for applying urine samples was used. The urine sample were transferred to the test strip through capillary action. If there wass appearance of reddish brown strip in the control region, it depicts that the test urine sample contains greater than or equal to 200ng/ml of cotinine. If the reddish brown strip appears in the test zone, the result was regarded as invalid. If one band appears in the control zone and one band appears in the test zone, this implies that the test was a negative result. However if a band appears in the test zone, the result was positive for nicotine. A sample of the nicotine test strip is shown in plates 3.2 and 3.4 respectively.

3.1.4 Commercially available cigarette (Aspen brand)

The cigarette of the Aspen variety has a label containing; nicotine 0.8mg carbon monoxide 10mg and tar 10mg respectively. It is manufactured by JTI (UK) Ltd for the JTI Macdonald corp. The standard for conversion from mg/m^3 to ppm is 1ppm = $1.45mg/m^2$ and $1mg/m^2$= 0.873ppm respectively. A sample of the aspen brand of cigarette is shown in plate 3.3.

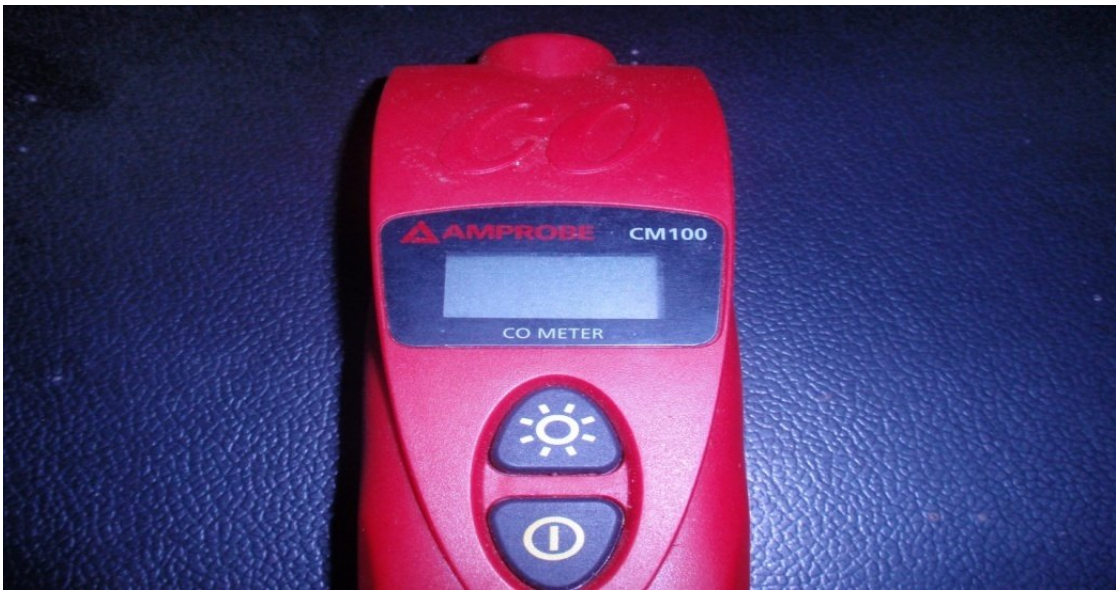

Plate 3.1: Amprobe Carbon Monoxide Meter

Plate 3.2: Nicotine screening strip

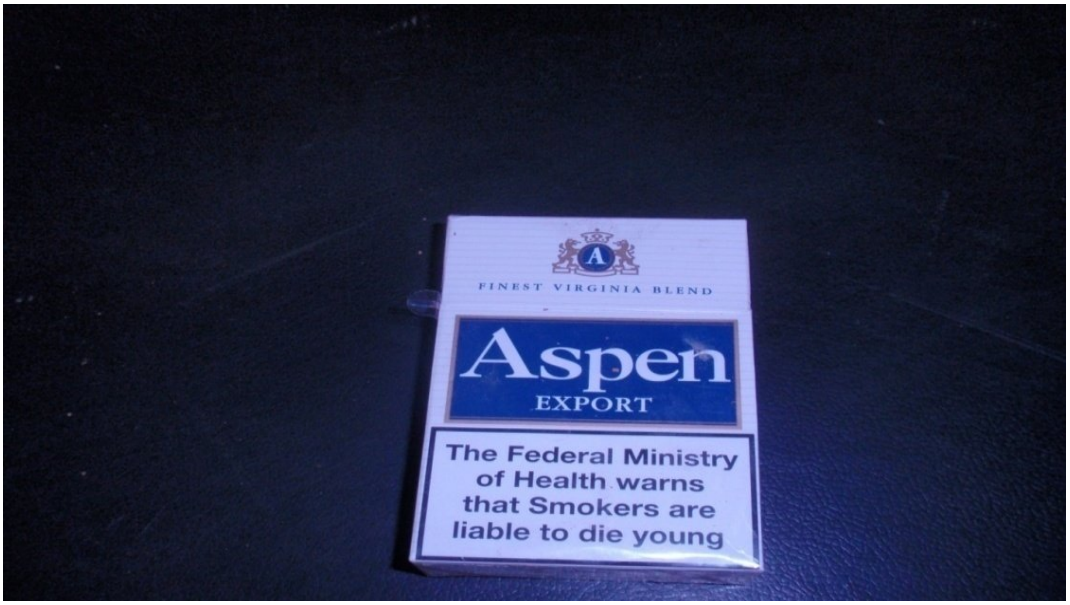

Plate 3.3: Commercially available cigarette (Aspen brand)

Plate 3.4: Package of the nicotine test strip with its brand name on it

3.1.5 Morris water maze training tank

The tank is of dimensions of 150 cm in diameter and 30 cm deep. The tank is painted black along with the 12 cm platform submerged about 2cm below the water level which is upto 20 cm of the 30 cm of the depth of the tank. The escape platform has a diameter of 12cm and is placed 12cm away from the west end of the tank, about 63 cm from the centre spot of the tank.

3.1.6 Fabricated chamber for static exposure

The chamber for the exposure is a tightly sealed rectangular transparent plastic container. The chamber measures 50 cm by 20cm by 20cm as shown in plate 3.5.

3.1.7 Microscope

An Axioskop 40, Zeiss light microscope which has binocular lens for focusing. The microscope is fitted with lenses for magnification of 5, 10, 20, 40 and 100 respectively.

Plate 3.5: Plastic Exposure chamber

Plate 3.6: Electric Incense burner

3.1.8 Neuber Improved Bright Lines Counting Chamber

The counting chamber is of 0.100mm depth and 0.0025mm2 area and made in Germany by Marienfeld.

Other materials include:

Incense burner, Petri dish and centrifuge tubes, Timer, Dissecting instruments; Scissors forceps; Surgical blade; needles; Weighing scale; Ph meter; Automated Water bath set at 37^0C; 10% Formal saline ; Bouins fluid; 2.5% Trypsin enzyme, HEPES buffer (catalogue number 15630) (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); N-(2-Hydroxyethyl) piperazine-N'-ethane sulfonic Acid); Hanks balanced salt solution (catalogue number 14185).

3.2 Method

Exposure to cigarette smoke was achieved by burning chops of cigarette sticks directly on a miniature incense burner as shown in plate 3.6 was kept within a plastic aquarium-like container in which the rats were housed during exposure to cigarette smoke. The incense burner kept was protectively separated from the animals by an iron mesh. The cigarette smoke was generated once the chops of cigarette stick were placed on the red hot plate of the incense burner. Since the Wistar rats are also in the exposure chamber which was sealed during exposure, the Wistar rats were therefore exposed to the carbon monoxide. The concentration of carbon monoxide was monitored on the digital screen of an Amprobe carbon monoxide meter to ensure that the Wistar rats were exposed to the range of 10-32ppm of carbon monoxide.

The exposure to cigarette smoke was done for one (1) minute, and then the exposure chamber was opened to allow ventilation with fresh air for 1min. This cycle of exposure and ventilation was carried out until a total of 20 mins of exposure was attained every day for four days in a week for 5 weeks. This method was designed in light of other exposure studies relating to cigarette smoke as carried out by Rajpurkar *et al*., (2000, 2002), Güven *et al*., (1999) and Ahmadnia *et al*., (2007). Nicotine test strip was employed in testing the presence of cotinine in the urine of the rats, a by-product of nicotine metabolism, to show that the Wistar rats actually inhaled the cigarette smoke.

A positive reaction on the cotinine test strip obtained following application of a drop of urine on to the test well was further interpreted as the presence of greater than or equal to 200ng/ul of nicotine in the blood.

3.2.1 Morris Water Maze Task

Each rat was placed in the water, tail first, immediately facing the perimeter of the tank at one of the cardinal compass points (north, south, east or west). In this case the east, and then was allowed a maximum time of 60 seconds to locate the hidden platform. Finding the platform was defined as staying on it for at least 2 seconds. If the subject failed to find the platform in the allotted time, it was placed onto the platform for 10 seconds and assigned a latency of 60 seconds. After each subject in the testing squad completes one trial, the next trial will be carried out the next day.

The training involved an initial pre exposure training conducted on daily basis for 3 days, aimed at acquinting the rats to the swimming environment and hence obliterate the modulating influence of stress in the future recordings for the Morris water maze task and

other confounding factors. The habituation training for three days was followed by the definitive training for three days, each subject having one training trials per day.

For the training periods, the platform is located to the northwest quadrant, where it was hidden from view by painting the tank and the platform black. The rats were timed during each training session and the latency time documented for each training. All the trainings were carried out during the day specifically from 2 pm and ended around 5 pm.

Only rats that performed the Morris water maze training without drowning or other unexpected reaction of the rats to the presence of water were used for the study. Both the experimental and the control rats were trained in the Morris water maze for 3 trainings over 3days.

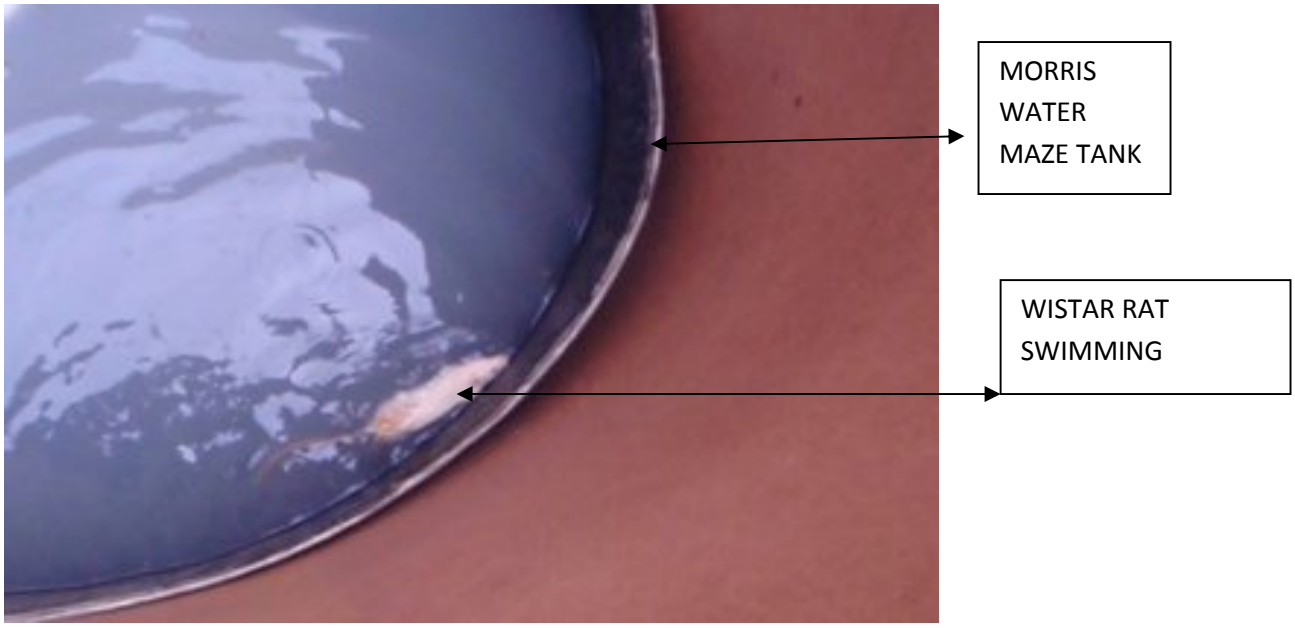

Plate 3.7: Morris water maze task being performed by rat.

Similarly, following a 20 minutes per day exposure to cigarette smoke maintained at 10 – 32ppm for 4 days every week for 5 weeks, the animals in the exposure groups were taken through the exposure regimen while the animals in the control group were placed in the exposure chamber without getting exposed to cigarette smoke but only inhaling the ambient air. The Morris water maze training was repeated for animals following the 5 weeks exposure to cigarette smoke and the repeat post exposure training was carried out daily for 3 days. The reference memory model of the Morris water maze task was employed in this work, which portends that as the animals perform each cycle of training, the latency/ reference time will continue to decrease. Plate 3.7 shows a Wistar rat performing Morris water maze task in a tank.

3.2.2 Tissue Preparation for Cell Suspension

The animals were placed in an inhalation box packed with cotton wools soaked in chloroform. The animals were allowed in the inhalational box for the shortest period of time necessary for the animals to become anaesthetized by the chloroform. The anaesthetized rats were then decapitated and immediately the heads to be dissected for tissue suspension were placed in Calcium and Magnesium free Hanks balanced solution (CMF- HBSS). While the heads to be dissected for histological slide preparation were placed in Bouins fluid for fixing the tissue. Careful dissection of the brain by dissecting away the mesencephalic region and then sectioning out the medial temporal lobe region of the brain. The medial temporal lobe region of the brain houses the hippocampus. Plates 3.8, 3.9 and 3.10 show stepwise dissection of the hippocampus of Wistar rat.

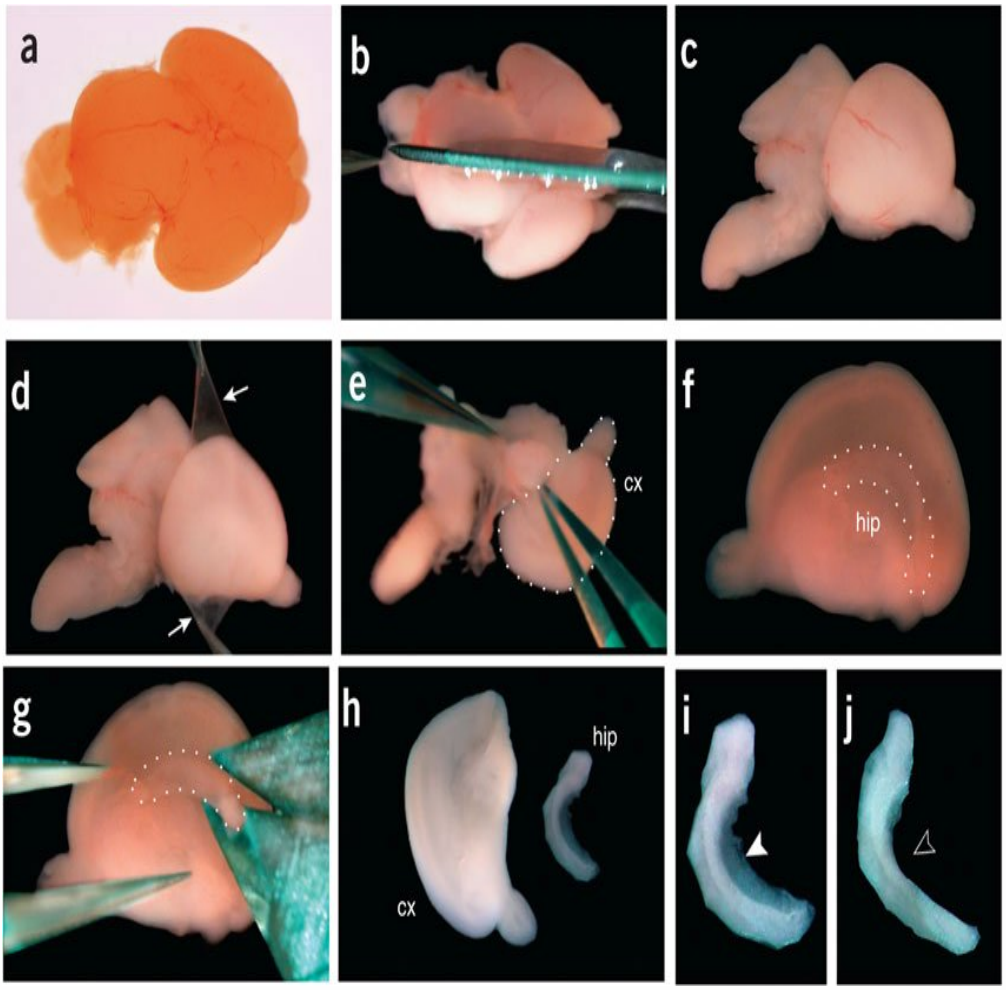

Plate 3.8: (**a**) The E16.5 mouse brain is (**b**) cut into halves via the midline sagittal section. (**c**) The halves are placed such that the cortex is facing up. (**d**) Meninges (arrows) are removed from the hemispheres with Dumont no. 5 forceps by gently pulling. (**e**) While holding the brainstem, the cortex (cx) is flipped outward and clipped off the midbrain. (**f**) The hippocampus (hip) is visible on the inner surface of the forebrain. (**g**) The hippocampus is removed with a microscissor. (**h**) Cortex (cx) and hippocampus (hip) are separated. (**i,j**) The fimbriae (arrowhead) on the concave side of the hippocampus are removed (open arrowhead).

Source: Thomas *et al*. (2008).

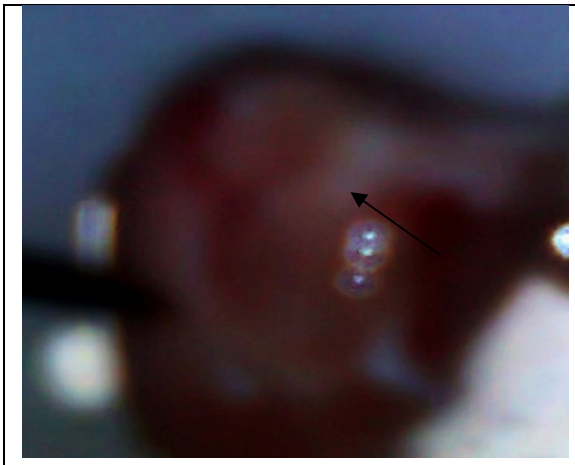

Plate 3.9: Medial half of brain of Wistar rats Showing hippocampus (long arrows)

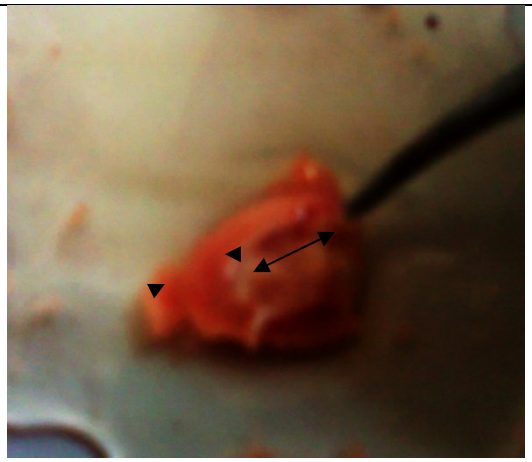

Plate 3.10: Medial half of brain of Wistar rats showing hippocampus been dissected (long arrows), olfactory lobe (arrow head)

3.2.2.1 Preparation of hippocampal cell suspension

The cell suspension was made through the following procedure:

1. Hippocampi were dissected out and collected in a petri dish containing Calcium and Magnesium free Hanks balanced solution (CMF- HBSS).

2. Frozen trypsin enzyme solution was allowed to thaw in refrigerator overnight at 4^0C and then incubated in a 37^0C automated water bath just minutes to using the solution.

3. The hippocampi were then transferred into a 15ml centrifuge tube and 4.5ml of CMF-HBSS was added along with 0.5ml of 2.5% trypsin. Incubated for 15 min in a water bath at 37^0C.

4. Trypsin was then gently removed and 5ml of CMF-HBSS was added and allowed to stand for 5min at room temperature to allow trypsin to diffuse out of the tissue. Then the solution was decanted leaving only the hippocampi tissues.

5. 2mls of CMF-HBSS was added to hippocampi tissues and the pippeted up and down to dissociate the tissue into solution of pyramidal cells.

6. Estimation of cell count in the cell suspension was achieved by applying a drop of cell suspension on to a Neuber hemocytometre counting chamber (Stefanie and Banker 2006).

Plates 3.11 and 3.12 shows the hippocampal suspension in a neuber counting chamber's frame

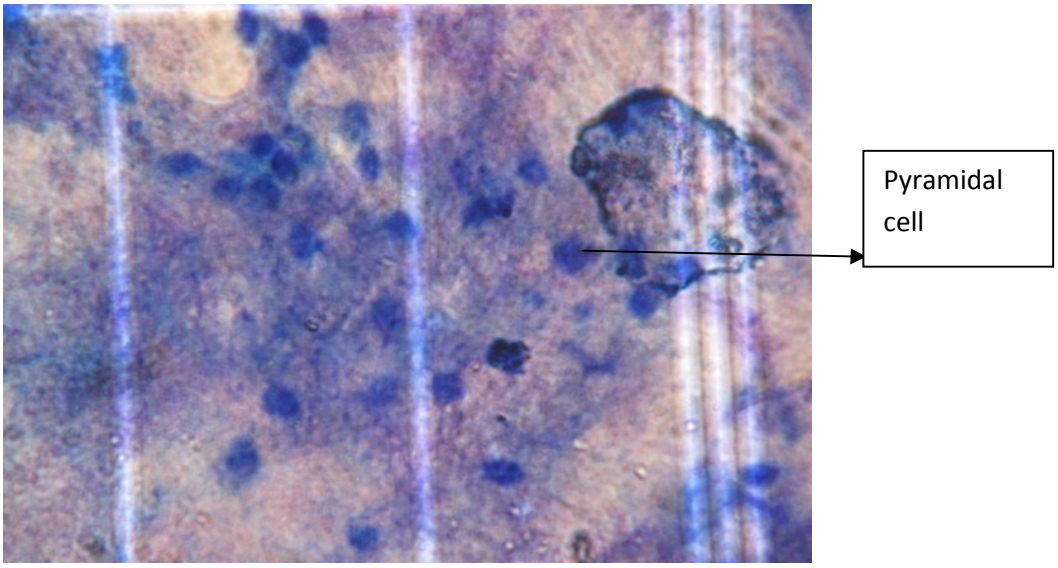

Plate 3.11: Counting grid filled with hippocampal cell suspension stained with dip quick for control animals (x 400).

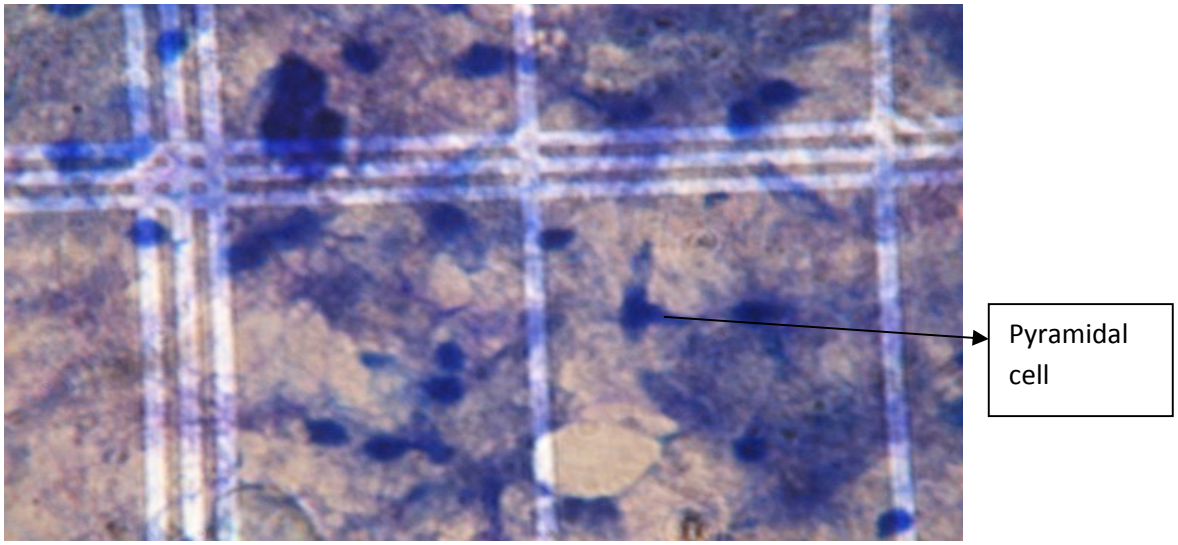

Plate 3.12: Counting grid filled with hippocampal cell suspension stained with dip quick for Experimental animals (x400).

3.2.3 Preparation of Histological Slides

The brain tissues were fixed in the bouins fluid and were processed for tissue section using the following method. Dehydration was done using graded alcohol levels, clearing using xylene and impregnation and infiltration in paraffin wax and finally embedding into tissue blocks.

The tissue was then sectioned using rotary microtome set at 15 microns. The sectioned tissue were then dewaxed and stained with Hematoxylin and eosin and Marsland and Glees Silver staining (see appendix for staining techniques).

A detailed inspection of corresponding slide of tissues (every 5^{th} section of the tissue) observed carefully under the guidance of a consultant cytologist for the presence of any pathology was carried out. Photomicrographs of the slides were obtained and the cells in the pyramidal layers of the slides at various regions were counted with the aid of the image j software manual cell counter. The type of cell identified for counting were the numerous normal appearing soma of pyramidal cell and the very few Pyknotic pyramidal cells respectively.

3.2.4 Assessment of the Sections of the Hippocampus

The slides were extensively studied for the following:

The cell number per high power field of the different regions of the hippocampus

The number of Pyknotic cells in different regions of the hippocampus per high power field.

3.2.5 Statistical Analysis

The data from the work will be presented as mean standard deviation. The SPSS 15 will be used to analyse the data. Student t test will be employed for the comparisons of means as appropriate. Significance is assumed when $P \leq 0.05$.

CHAPTER FOUR

4.0 Results

A total of 30 adult Wistar rats were used in this study, from which 12 (40%) were females and 18 (60%) were males. The males and females were then randomized into 10:5 for the control and 8:7 for the experiment respectively. The averages of the latency times of the Morris water maze training (MWMT) recorded for each of the rats were obtained for both the pre exposure and post exposure training respectively. Similarly, the mean and standard deviations for the differences for the pre exposure and post exposure timing were obtained.

4.1 Physical observations

Following two weeks of acclimatization, the wistar rats were noticed to be calm and responding appropriately to stimuli. During the first Morris Water Maze training, the rats showed little attempt at locating the escape platform rather, the rats showed more effort in trying to jump directly out of the tank. During exposure to cigarette smoke, the rats were always trying to crowd themselves away from the source of cigarette smoke. Even after the exposure period, the rats vigourously cleanse their fur by scrubbing the fur with saliva on their tongues. Following the exposure to cigarette smoke, the rats transform progressively from calm quite rats to aggressive wild rats. Weights of the Wistar rats were recorded and the characteristic spread was displayed in table 4.1, 4.2 and figure 4.1, 4.2 respectively. There was a significant decrease ($P \leq 0.05$) in the weight of the rats in the experimental group and female group compared to the control and male group respectively.

Table 4.1: Shows the Weight by group of the rats

Variables	Experimental wistar rat (n=15)	Control wistar rats (n=15)	T	p
Weight (g)	244.67± 20.66	222.00 ± 27.31	2.56	0.016
Range(g)	224-265	190-250		
Gender (M/F)	8/7	10/5		

(Values are mean ± standard deviation, n = number of rats, weights compared using student t- test with P≤0.05)

Table 4.2: Shows the Weight by sex of the rats

Variables	Male wistar rat (n=18)	Female wistar rats (n=12)	T	p
Weight (g)	240.00± 21.96	215.00 ± 35.03	2.41	0.020
Range(g)	218 -262	180 -250		

(Values are mean ± standard deviation, n = number of rats, weights compared using student t- test with P ≤0.05)

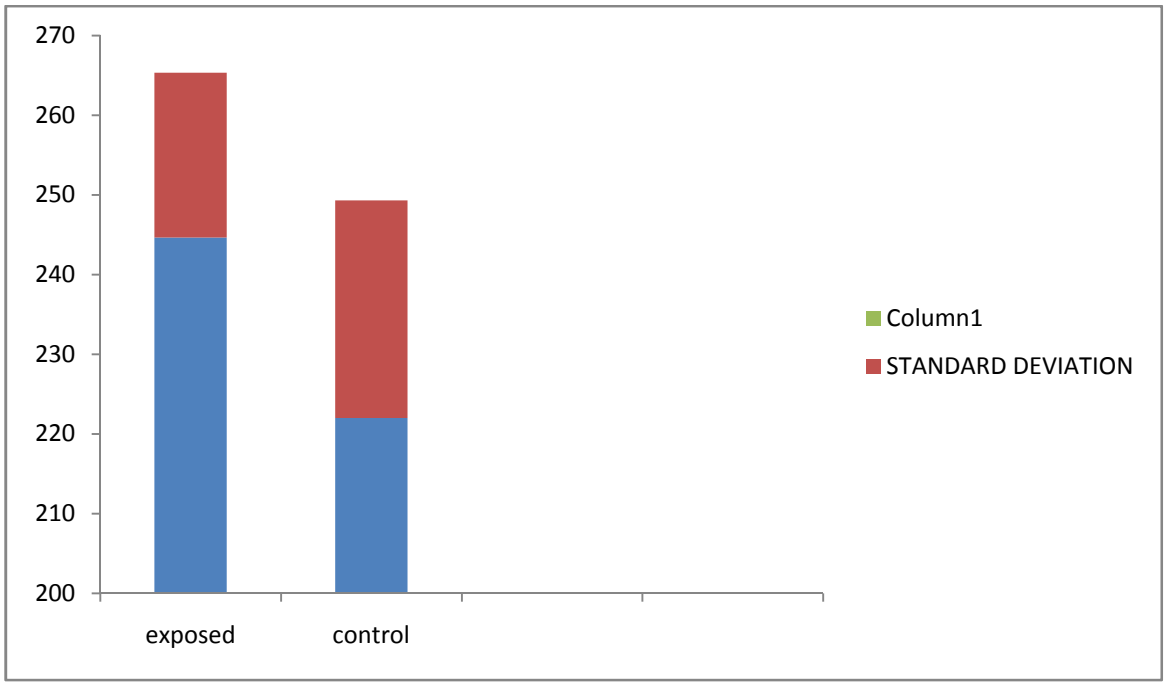

Figure 4.1: The weight distribution and the standard deviation of the wistar rats based on study grouping

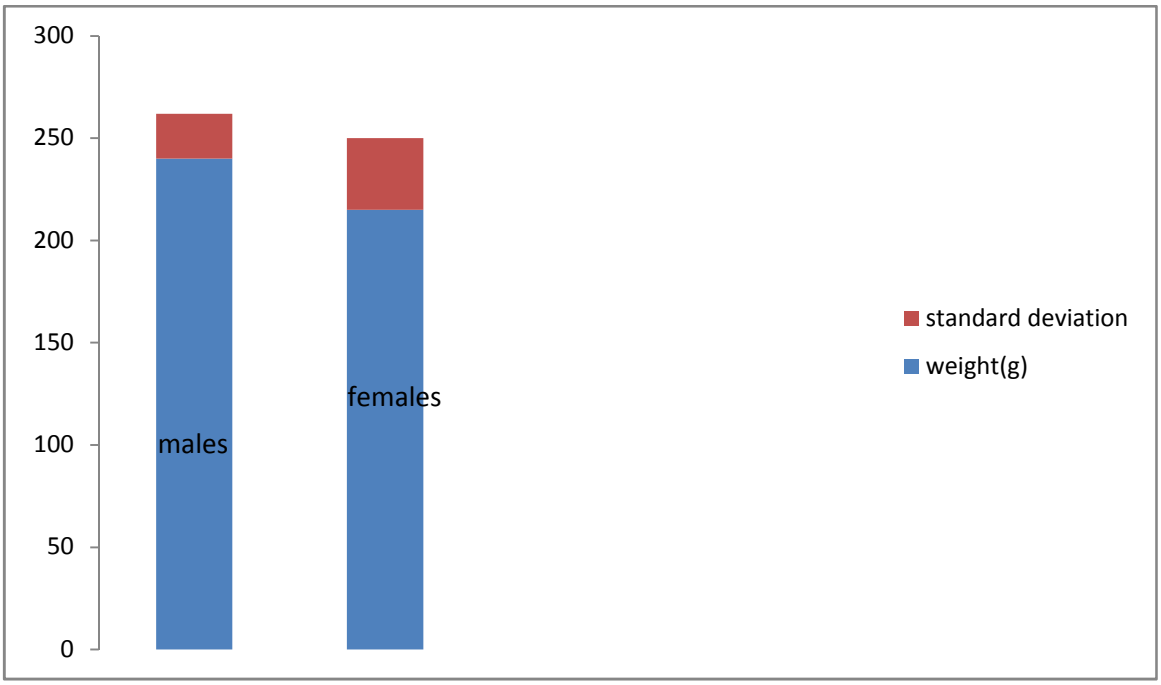

Figure 4.2: The weight distribution and the standard deviation of the wistar rats based on sex

4.2 Morris water maze

Table 4.3 presents the latency times for the pre-exposure period and the post exposure periods of the control animals with respect to gender. There was a significant decrease ($p \leq 0.05$) in the reference time of the Morris water maze training of the Male rats in the control group. There was a slight decrease in the latency periods between the early and late MWM timings of the female rats though this decrease was not statistically significant difference. Similarly there was no significant decrease in the latency times for the morris water maze task (MWMT) between male and the female for the early and late time recordings. Table 4.4: Summarizes the difference in Morris water maze task timing for experimental animals both before and after the exposure period, in relation to the gender of the rats. For the males and the females, there was no significant difference. The difference in the MWMT of the early and late timing of the male to female in the MWMT was not statistically significant.

4.3 Cell count of the Hippocampal cell suspension

Table 4.7 presents the difference between the cell count of the experimental rats to the control rats respectively which was not statistically significant ($P \leq 0.05$).

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Table 4.3: Early and Late time of the control male and female rats

Variables	Male rats (n=10)	Female rats (n=5)	t	df	P
MT1(S)	13.88±7.18	11.89±5.92	0.53	13	0.60
MT2(S)	7.93 ± 3.39	9.56 ± 3.89	-0.84	13	0.42
P	0.03	0.56			

(Values: mean ± standard deviation, MT1 and 2(S) =Maze Time(Sec). n = number of rats.

P≤0.05).

Table 4.4: Early and Late timing of the male and female groups of the experimental animals before and after exposure

Variables	Male rats (n=8)	Female rat (n=7)	T	Df	P
MT1(S)	10.84±5.78	13.83±9.41	-0.76	13	0.46
MT2(S)	9.60±7.19	13.68±8.37	-1.02	13	-0.32
P	0.75	0.97			

(Values are mean ± standard deviation. MT(S) =Maze Time (Seconds) n = number of rats. P≤0.05)

Table 4.5: T test of the cell counts on tissue sections of the male rats of the control and experiment animals respectively.

Variable	Control (n=5)	Exp (n=5)	Df	t	P
CCOUNT	241.60±17.87	219.80±16.05	8	-2.029	0.08

(Ccount = Cell count, Value is mean ± standard deviation, n= total number of wistar rats. T test $P \leq 0.05$)

4.4 Cell count of tissue section

One of the objectives of the study was to compare tissue sections from Male animals in the exposed groups and those in the control for any significant pathological changes in the hippocampus following exposure to cigarette smoke maintained at 10 -32 ppm of carbon monoxide. Tables 4.5 and 4.6 display the statistical findings obtained on counting the soma of pyknotic and normal pyramidal cells in the pyramidal layer of the hippocampus of the experimental or exposed groups and the control groups respectively. The difference between the normal pyramidal cell count of the experimental and the control slides of the CA1 region and CA2 region respectively is significant ($P \leq 0.05$) as revealed in table 4.6. Similarly, the difference between the pyramidal cell count of the experimental and the control tissue sections of the CA1 region is not statistically significant ($P \leq 0.05$) as revealed in table 4.5. while as there was a significant increase in number of the Pyknotic pyramidal cell of the experimental animals in both the CA1 and CA2 regions with ($P \leq 0.05$) as shown in table 4.7 Similarly, plates 4.1, 4.2, 4.3 and 4.4 respectively show the overview of the hippocampus with peculiar findings for the exposed group of rats of increased pyknotic cells (plate 4.4).

Table 4.6: Normal pyramidal cell count (Ncc) in CA1 and CA2 region of male rats

Variables	Exposed wistar rat (n=5)	Control wistar rats (n=5)	T	p
Ncc1 (unit)	82.4± 2.07	75.2 ± 1.30	6.57	0.00
Ncc2(unit)	83.2± 1.64	75.2 ± 1.30	6.57	0.00

values are mean ± standard deviation, n = total number of wistar rats, t test with P>0.05

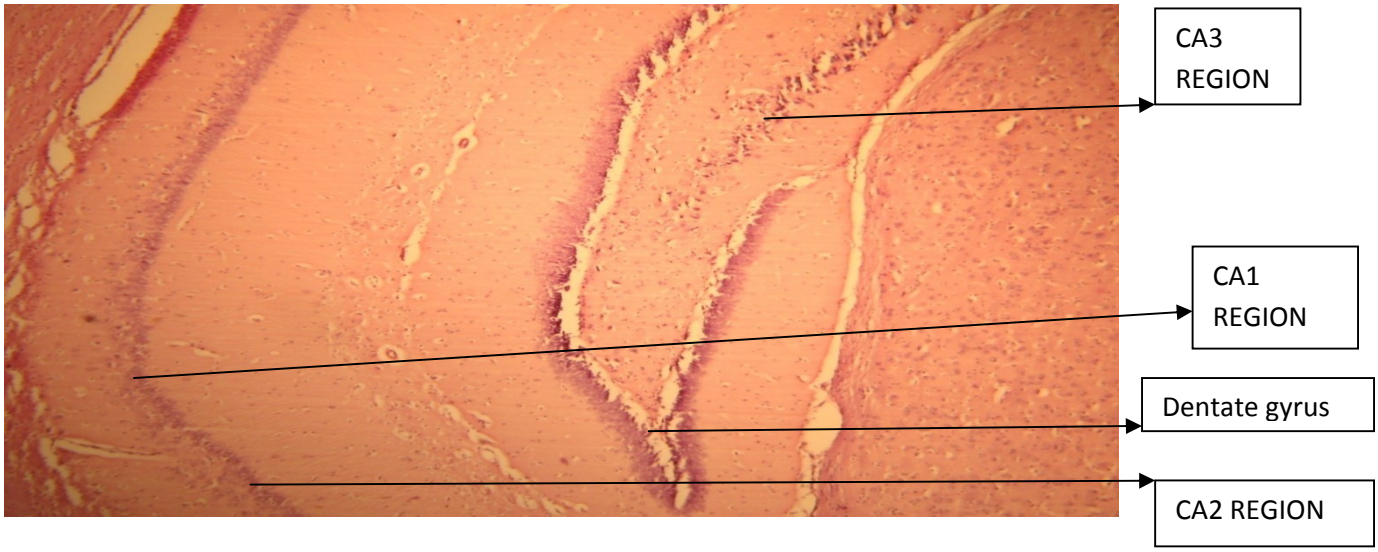

Plate 4.1: Overview of the hippocampus of the control Wistar rats (x400) showing dentate gyrus,CA1, CA2, CA3 regions respectively.

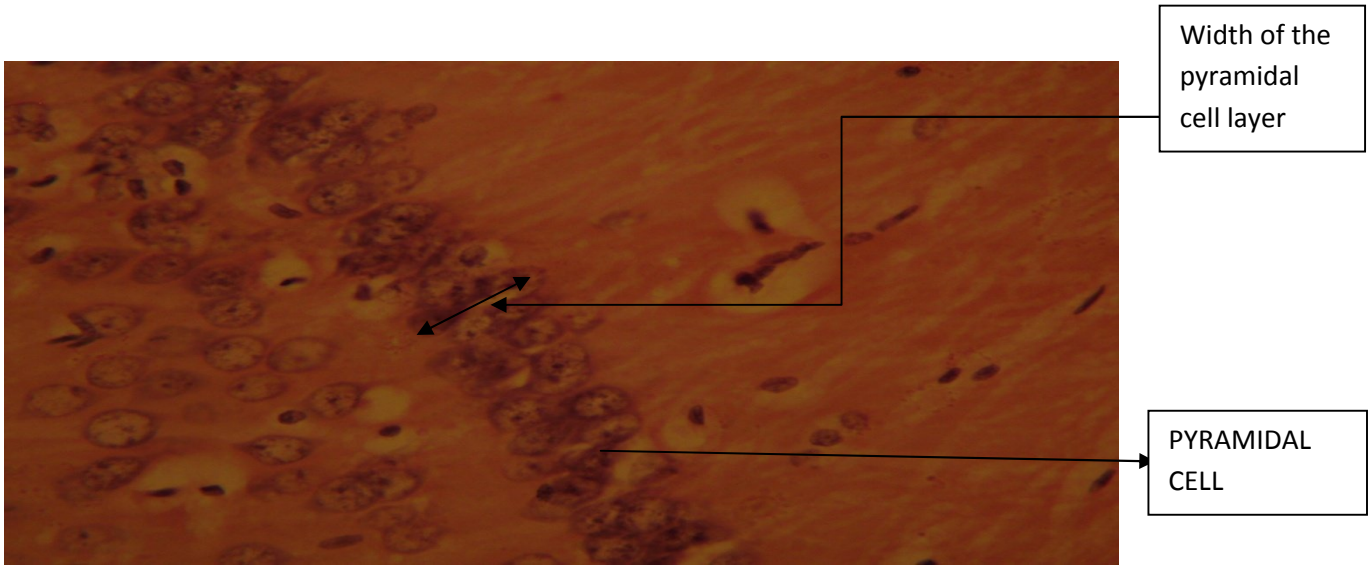

Plate 4.2: A section of CA1 region of the hippocampus of the control Wistar rats (x400) showing pyramidal cells and narrow width of the pyramidal cell layer.

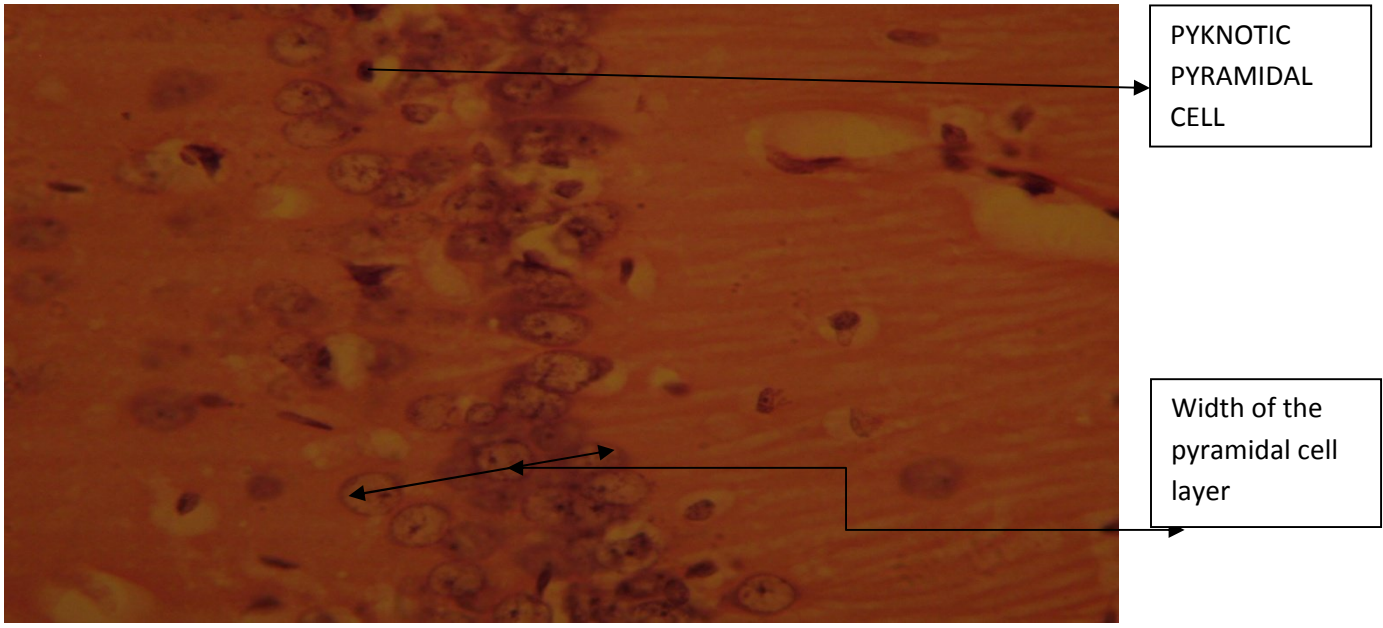

Plate 4.3: A section of CA2 region of the hippocampus of the control Wistar rats (x400) showing pyramidal cells and wide width of the pyramidal cell layer.

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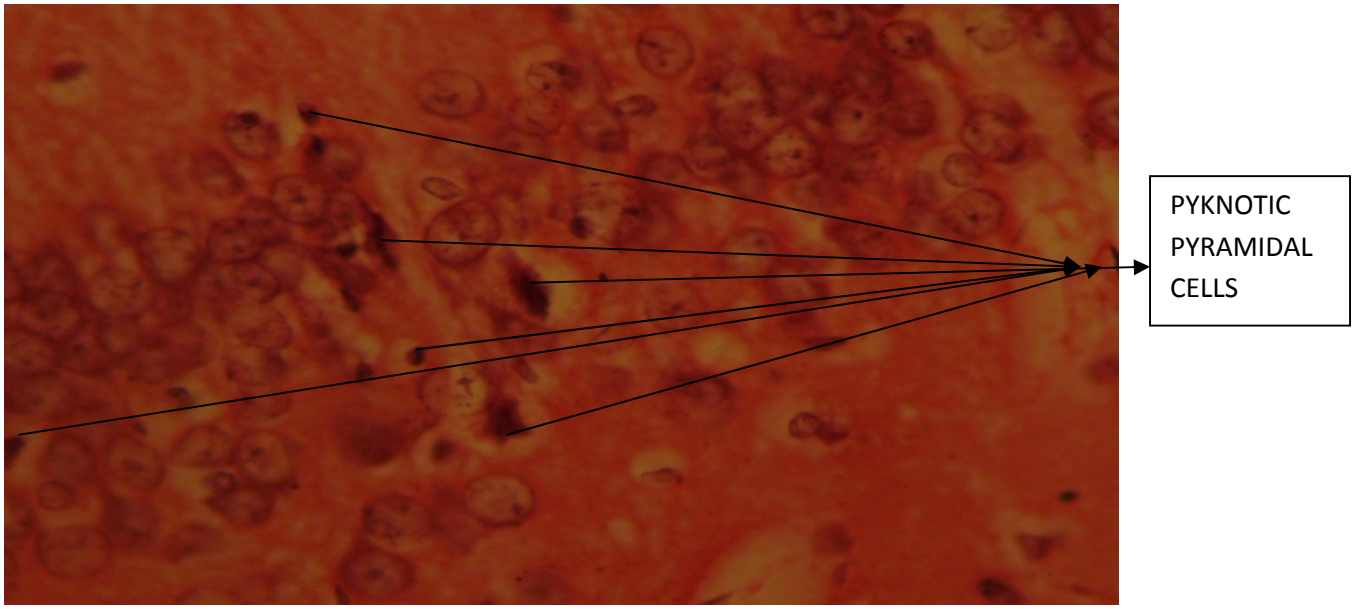

Plate 4.4: A section of CA1 region of the exposure Wistar rats (x 400) showing pyknotic pyramidal cells.

Table 4.7: Pyknotic cell count of study group (Pcc) in CA1 and CA2 region male rats

Variables	Exposed wistar rat (n=5)	Control wistar rats (n=5)	T	p
Pcc1 (unit)	10.8± 1.30	4.2± 1.30	8.00	0.00
Pcc2 (unit)	16.4± 1.67	10.0± 1.22	6.90	0.00

(Values are mean ± standard deviation, pcc: pyknotic cell count. T test with $p \leq 0.05$)

4.5 Histological Observations of tissues stained with Marsland and Glees (M&G) silver stain

The architectural outline of the neurons appear normal with distinct nerve fibers seen in both the exposed and control slides. However the perikaryal swelling and the Pyknotic cells are seen with this staining as shown in plates 4.5 and 4.6..

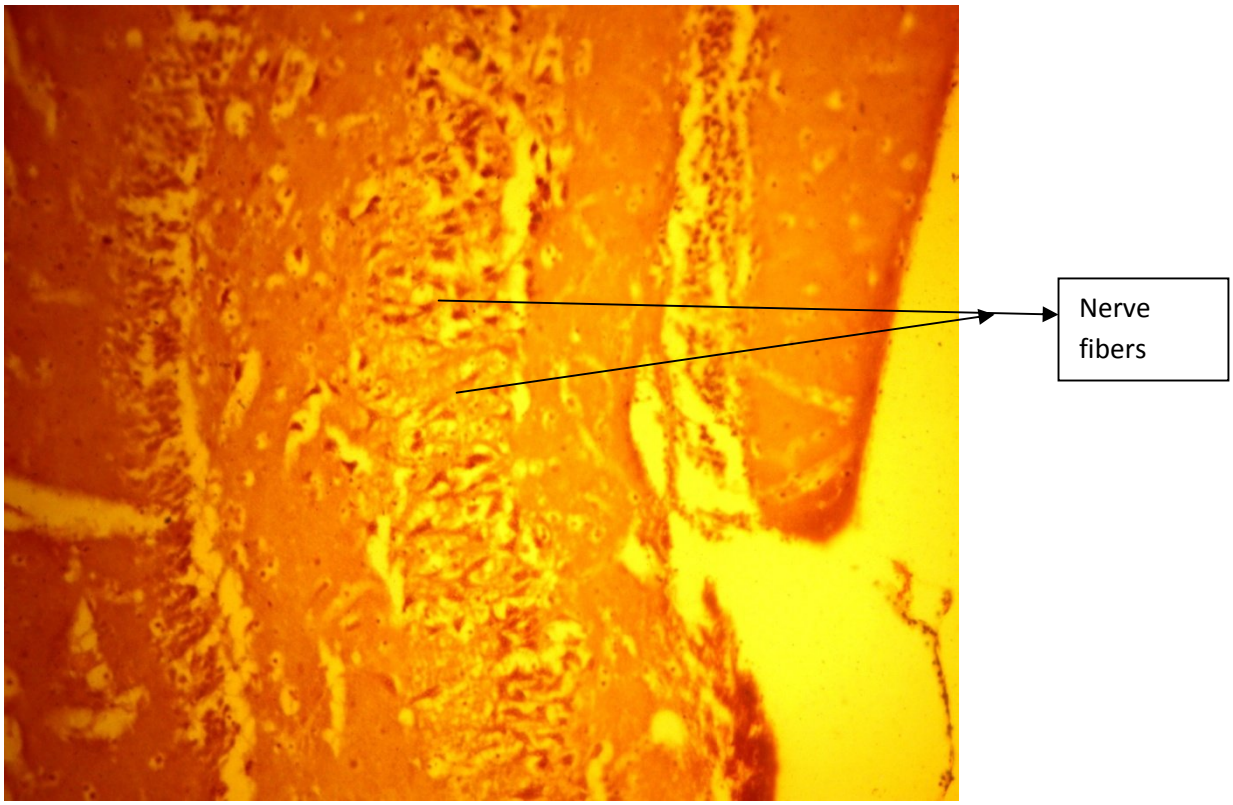

Plate 4.5: A section of hippocampus of control Wistar rats, M & G Stain (x400) showing nerve fibres.

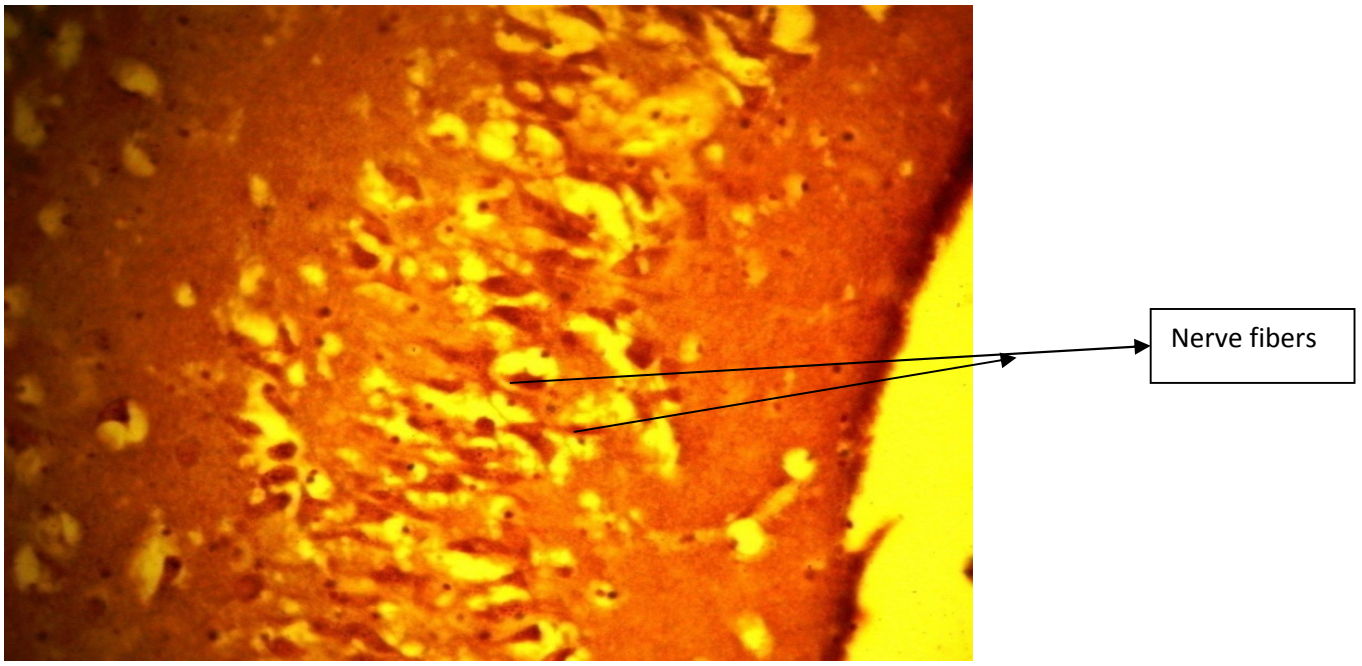

Plate 4.6: Section of hippocampus of experimental rats, M&G stain (x400) showing nerve fibres.

CHAPTER 5

5.0 DISCUSSION

The main objective of this study was to compare the structural and functional effects of exposure to cigarette smoke maintained at 10-32 ppm of carbon monoxide on the hippocampus of Wistar rats. The outcome of this study reveals the following:

5.1 Physical characteristics

The behavior of the Wistar rats in the experimental group was mildly aggressive with gathering or crowding away from the source of cigarette smoke. The experimental rats vigorously tried to rub off the cigarette smoke off their furs, suggesting that the cigarette smoke was not pleasant to the animals.

5.2 Morris water maze

The performance of Morris water maze task assessed was not significantly improved in male and female Wistar rats exposed to cigarette smoke maintained at 10 - 32 ppm of carbon monoxide. Control female Wistar rats reveal no statistically significant difference in the time records obtained initially and after 5 weeks of delay without MWMT. However there was a significant decrease in the Morris water maze reference time between the early and the late MWM task of the male control rats.

The above result implies that the males tend to learn and retain some of the learned information (spatial memory in the context of the present work) for a sufficiently longer time than in the female. Hence providing the edge for the males to out perform the females in the MWMT. Interestingly this result is in keeping with the work of Cimadevilla *et al*. (2004) which showed that in spatial memory assessment, the female rats tend to do poorly

especially if there is a delay of as little as 24hours before the next task is done (Cimadevilla *et al*., 2004).

Similarly, regarding the disruptive effect of cigarette smoke on the performance of the MWMT in the experimental rats, researches have shown that, in smokers and patients suffering from a variety of neuropsychiatric disorders, nicotinic agonists act beneficially on several aspects of cognition, including working memory attention, learning, and memory. In fact, nicotinic treatments are being developed as therapy for cognitive dysfunction in disorders such as Alzheimer's disease, Parkinson's disease and schizophrenia (Levin *et al*., 2005; Newhouse *et al*., 2004a; Newhouse *et al.*, 2004b) and (Picciotto and Zoli, 2002). In contrast, in normal nonsmokers, nicotine tends to have deleterious effects on cognitive performance (Newhouse *et al*., 2004b). The present work on the effect of cigarette smoke maintained at 10-32 ppm of Carbon monoxide on the structure and function of the hippocampus of Wistar rats appeared to be in consonance with the findings of Newhouse *et al.* (2004b). Hence the experimental animals both males and females showed no significant decrease in their performance time in the Morris water maze task just like the control female. But the control male rats showed significant decrease from the high time record initially to the significantly lower time record finally.

5.3 Cell count

5.3.1 Cell count for hippocampal suspension

There was no significant difference in the cell count in the hippocampal cell suspension of male Wistar rats between the experimental and control Wistar rats respectively.

5.3.2 Cell count for tissue sections

There was a significant increase in the normal pyramidal cell counts in the experimental male Wistar rats compared to the control male Wistar rats in both the CA1 and CA2 regions. Likewise there was a significant increase in the pyknotic pyramidal cell count in the male Wistar rats in both the CA1 and CA2 regions of the tissue section of the exposed male rats compared to the control male rats.

5.3.3 Cell count

In a study involving exposure of a group of rabbits to cigarette smoke and another group of rabbits to Caffeic acid phenethyl ester (CAPE) just before cigarette smoke exposure, Olcay *et al*. (2007) were able to show that the rabbits exposed to cigarette smoke alone had an increase in the population of the apoptotic bodies in the CA1, CA2, CA3 and dentate gyrus areas than the rabbits that were treated with CAPE, even though they also received cigarette smoke. Hence the CAPE compound confers a neuro-protective effect on the neurons of the hippocampus against the changes induced by the cigarette smoke (Olcay *et al*., 2007). Zoli *et al*. (1999), showed that stimulation of nicotinic cholinergic receptors in mature cells can actually decrease the cell death elicited by injurious treatments potentially by induction of neurotrophic factors (Maggio *et al.,* 1998). While Trauth *et al*. (2000) showed that adolescent nicotine treatment evoked decreases in total cell number as assessed by DNA measurements in cerebral cortex, midbrain and hippocampus.

Clearly then, the developmental stage at which nicotine exposure occurs was critical in determining the outcome. The Pyknotic cell count in the tissue sections was in keeping with the findings of the work of Olcay *et al*. (2007), since tissue sections from experimental animals showed a significant increase in the population of Pyknotic pyramidal cells unlike the sections from the control rats. While, the significantly higher count of normal pyramidal cell count in the experimental tissue sections was contrary to the findings of the work of Trauth *et al.* (2000) which showed a decrease in the number of the normal pyramidal cells in the hippocampus of adolescent rats following exposure to nicotine. It was uncertain why there should be departure from the other works, but some hypothesis could be put forward. This could probably be that at low doses of cigarette smoke maintained at 10-32 ppm of CO, there may be some unexplained stimulus for increased neurogenesis in the exposed rats as reported by Zoli *et al*. (1999). Hence implying that probably at this concentration of carbon monoxide in the cigarette smoke, neurogenesis was stimulated in the CA1 and CA2 regions of the hippocampus.

5.4 Summary and conclusion

5.4.1 Summary

This study investigated the effect of cigarette smoke maintained at 10 – 32 ppm of carbon monoxide on the hippocampus of Wistar rats. The overall mean weight of the study rats was 233.34gram with a range of 209.35 to 257.33, the rats were randomized into two groups of control and experiment which was further grouped into male and female groups: control male (CM), control female (CF), experimental male (EM) and experimental female (EF)

respectively. The weight difference in the rats for the experiment group showed significant difference, however no significant difference in the weight of the control rats.

Similarly, early and late reference times for Morris water maze task for the control and the experimental rats showed significant difference for the control males only. While there was no significant difference between reference Morris water maze times for the control female rats and the experimental male and female rats respectively. And also no significant difference across the Morris water maze task time for the early and late times across gender. Finally, there was significant difference in the cell count in the tissue section of the hippocampus of the control and exposed male rats for both the normal and Pyknotic pyramidal cells respectively.

5.4.2 Conclusion

These findings conclusively suggest that the effect of cigarette smoke maintained at 10-32ppm of CO leads to:

(1) Disruption of learning / memory for the MWMT in male experimental wistar rats.

(2) No beneficial effect to the learning / memory of the MWMT in the female experimental Wistar rats.

(3) The early MWMT training was sufficient enough to instill some significant improvement in the reference times obtained for the late MWMT in the male control rats but not in the female control rats. Suggesting further that the Males learn faster than the females in the Morris water maze task.

(4) The cell count from sections of the hippocampus show a significant increase in the proportion of normal and pyknotic pyramidal cells in the experimental compared to control rats.

(5) The cell count from the hippocampal suspension showed no significant difference between the samples of the control and experiment male rats.

5.5 Recommendations

The following recommendations are made based on the outcome of this study.

1. There is a need for further studies with a larger sample size for the chronic effect of the cigarette smoke maintained at 10 -32 ppm on the hippocampus.

2. Another area that might be of interest is, incorporating the use of probe time to ascertain acquisition of memory for the spatial activity instead of the reference time alone.

3. A similar research is recommended to be carried out using rats of approximately same weight so as to obviate any effect the weight of the rat may have.

4. There is a need for a similar study to be carried out utilizing advanced methods of objective unbiased cell counting.

5. There is a need for a similar study to be carried out utilizing staining techniques specific for apoptotic bodies

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APPENDIX

Hematoxylin and Eosin staining:

(1) Dewax and hydrate the tissue

(2) Stain in Mayers solution for 30 minutes

(3) Wash thoroughly in tap water

(4) Differentiate in 1%hydrochloric acid until only the cell Nuclei retain the stain

(5) Blue in scott's tap water for 5- 10 min

(6) Counter stain in eosin solution for 2 min.

(7) Wash in running water

(8) Dehydrate,clear and mount in DPX.

Result: Nuclei – blue., Cytoplasm – Pink

Marsland and Glees staining

(1) Treat sections with xylol for 1 minute to remove wax.

(2) Flood with alcohol for 30 seconds

(3) Flood slide with 1% celloidin for 20 seconds

(4) Wipe off excess celloidin from back of slide and flood with 70% alcohol

(5) Rinse in distilled water

(6) Treat with 20% silver nitrate at 37^0C for 30 minutes.

(7) Rinse in distilled water

(8) Flood with 10% formalin in tap water

(9) Impregnate with ammoniacal silver for 30 seconds

(10)Drain off silver solution and flood slide with changes of 10% formalin for 1 minute each.

(11) Fix in 5% sodium thiosulphate for 5 minutes

(12) Wash in tap water

(13) Blot and flood with absolute alcohol to remove celloidin film. The metallic precipitate which often occurs is usually confined to the celloidin film and is removed with it.

(14) clear in xylol and mount with DPX.