SYNAPTOGENESIS AND SPINOGENESIS OF ADULT HIPPOCAMPAL NEUROGENESIS IN LABORATORY LONG-EVANS RAT EXPOSED TO ENRICHED ENVIRONMENT.

BY

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DECLARATION

I, Dr Uzokwe Chioma Blessing hereby declare that this thesis is my work. It is being submitted for the degree of a Master of Science in Medicine (Anatomical Sciences/Neuroscience) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature.....

Date: 08th December , 2016

DEDICATION

To the moral venture of the insatiable quest of the human mind to better the existence of the human race through the quest for knowledge.

Smiling angel, 2016

To God, Almighty for His grace and divine strength, to carry through and to the *still small voice* that continually encouraged me through the dark and low moments of this pursuit.

To my mother, Comfort C Uzokwe for instilling in me much diligence, and reiterating the benefit of hardwork each time I wanted to give in. To my loving husband Jerry N. Ndefo for your support and to my precious daughter Sonia, for staying up with me those nights, smiling when I came to fetch you late and just being you at loving me tirelessly. Thank you!!!

PRESENTATIONS ARISING FROM THIS STUDY

1. Research design presented at the International Brain Research Organization (IBRO) Neuroscience School, Nairobi Kenya, December 2014.
2. Uzokwe CB, Nkomozepi P, Manger PR, and Ihunwo AO (2016) Pattern of Doublecortin positive neurones in the dentate gyrus of Long-Evans rats exposed to environmental enrichment. Poster presented at the Faculty of Health Sciences Research Day, University of the Witwatersrand 1st September 2016.

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/55/B

APPLICANT: Dr CB Uzokwe

SCHOOL: Anatomical Sciences

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *Synaptogenesis and spinogenesis in adult hippocampal neurogenesis in laboratory rodents (Long-Evans rat) exposed to enriched environment and wild rodents of the rain forest and the desert*

Number and Species

18 Rattus Norvengicus (Long-Evans Rats)

Approval was given for to the use of animals for the project described above at an AESC meeting held on 28 October 2014. This approval remains valid until 27 October 2016.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____ Date: 4th November 2014
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 4th November 2014
(Registered Veterinarian)

cc: Supervisor: Profs AO Ihunwo & PR Manger
Director: CAS

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ABSTRACT

This research studied adult hippocampal neurogenesis in the dentate gyrus of the hippocampus of the Long-Evans rat. Eighteen male Long-Evans rats were exposed to complex enriched environment, the running wheel environment for exercise as single influencing factor and the standard laboratory environment for 28 days. Thereafter the rats were transcardially perfused with 0.9 normal saline followed by 4% paraformaldehyde. The brains were removed and frozen sagittal sections cut at 50 µm. Brain sections were stained with Cresyl violet for cytoarchitecture. Immunohistochemistry and immunofluorescence techniques were employed for the immature neurons with defined processes using the marker doublecortin (DCX), neuronal proliferation marker Ki-67, the synapse marker, synaptophysin and the dendritic spine marker, synaptobrevin. Giemsa staining was used to identify pyknotic neurons followed by counts for DCX, Ki-67, pyknotic positive cells, and volume density of the dentate gyrus. Results indicated a statistically significant increase in brain weight ($p=0.5$) for the complex enriched group when compared to the running group and control. The typical cytoarchitecture of the hippocampus in rodents was observed with more densely packed granule cell layer in the dorsal limb of the dentate gyrus compared to the ventral limb especially in the enriched group. The Ki-67 immunopositive cell number between groups showed a variable difference with a three-fold increase each between the standard control and exercise, and between the exercise and enriched but a six-fold increase between the standard control and the complex enriched groups. Comparing the DCX immunopositive results, we observed also that the neuronal numbers, structure, dendritic patterns as well as the neuronal arrangement on the dorsal and ventral limbs of the dentate gyrus varied significantly among groups. The apoptotic cell numberusing pyknotic cells, showed the standard control group to have the highest number of cells compared to the exercise versus the enriched group; noting a five-fold difference between the standard control and exercise, a twenty seven-fold difference between the standard control versus enriched and a twenty one-fold difference between

the exercise and complex enriched group. The volumetric analysis showed a 15-fold difference between the standard control and exercise groups, a five-fold difference between the exercise and complex enriched and a nineteen-fold difference between the standard control and complex enriched groups. However, no statistical significant difference was found in the volumetric analysis of the dentate gyrus between the groups.

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CHAPTER ONE: INTRODUCTION

1.1 Adult neurogenesis

Neurogenesis is the process by which neurons are generated from neural stem cells and progenitor cells (Alexander et al, 2013). Neurogenesis is most active during pre-natal development, and responsible for populating the growing brain with neurons. However, adult neurogenesis entails making functional neurons from precursors, which was conventionally reported to occur during embryonic and perinatal stages in mammals (Ming and Song, 2005). Altman et al (1965) furnished the first anatomical evidence for the presence of newly generated dentate granule cells in the postnatal rat hippocampus. The functional integration of new neurons in the adult central nervous system (CNS) was first shown in songbirds by Paton and Nottebohm (1984). Reports showed that multipotent neural stem cells were subsequently derived from the adult mammalian brain (Reynolds and Weiss, 1992 and Richards et al., 1992). The 'field of adult neurogenesis' gained a lot of momentum after the initiation of bromodeoxyuridine (BrdU), a nucleotide analogue, as lineage tracer (Kuhn et al., 1996) aiding demonstration of life-long unceasing neurogenesis in almost all mammals examined, including humans (Eriksson et al., 1998).

Driven by general interest and aided by methodological advancements, huge progress has been recorded over the past decade in the study of each and every aspect of adult neurogenesis in the mammalian CNS. Active adult neurogenesis is spatially restricted in normal conditions to two primary "neurogenic" brain sites, the subgranular zone (SGZ) within the dentate gyrus of the hippocampus, where new dentate granule cells are generated; and the subventricular zone (SVZ) of the lateral ventricles, where new neurons are generated. These new nerons then migrate through the rostral migratory stream (RMS), reach the olfactory bulb to become interneurons (Figure 1A) (Gage, 2000). Adult neurogenesis is a dynamic, finely tuned process and is subject to alterations by various physiological, pathological, and pharmacological stimuli. Neurogenesis in other adult

CNS regions is largely believed to be very limited under normal physiological conditions but could be induced after injury (Gould, 2007), exercise and environmental enrichment (van Praag et al, 1999).

A great deal has been learned about identities and properties of neural precursor subtypes in the adult CNS, the promoting local environment, and the serial steps of adult neurogenesis, which ranges from neuronal precursor proliferation to synaptic integration of newborn neurons (Alvarez-Buylla and Lim, 2004, Duan et al, 2008 and Lledo et al, 2006). Studies also have begun to illustrate the functional impact of new neurons on the existing neuronal circuitry and their contributions to brain functions under both the normal and disease states (Deng et al., 2010). These research areas being very rewarding, have not only provided important answers to many fundamental questions about adult neurogenesis but also making gross impact on general principles of stem cell regulation, neuronal development, structural plasticity, and disease mechanisms (Ming and Song 2011).

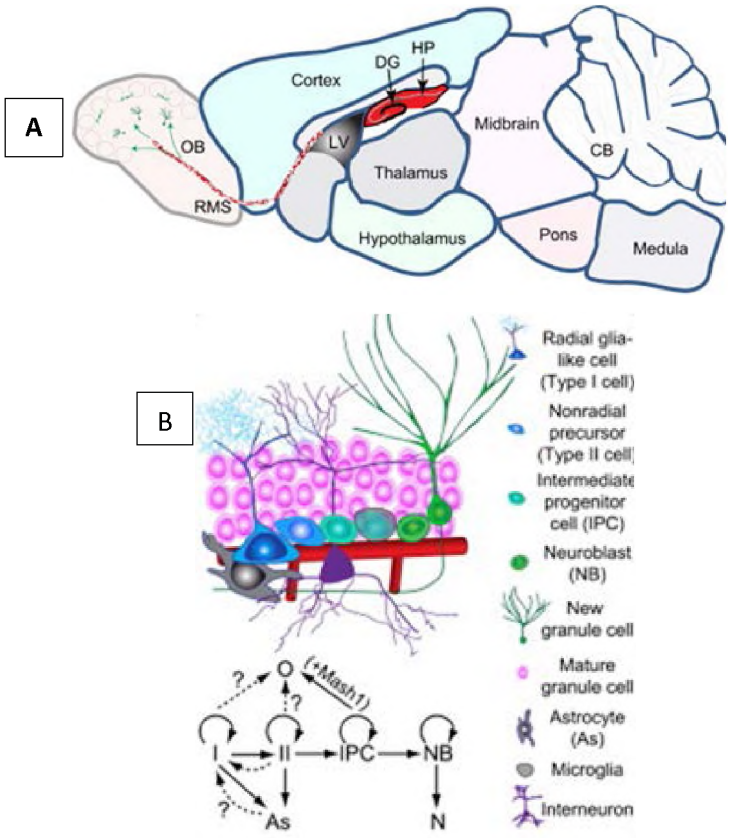


Figure 1.1: Models of the rat brain and neural stem cells with their lineage relationship in the Adult Dentate Gyrus. Gage (2000)

(A) Sagittal section of an adult rodent brain highlighting restricted and constant regions, exhibiting adult neurogenesis: dentate gyrus (DG) in the hippocampus (HP), and the subgranular zone (SVZ) of the lateral ventricle (LV) to the rostral migratory stream (RMS) of the olfactory bulb (OB).

(B) A schematic illustration of the neural stem cell niche in the subgranular zone (SGZ) in the dentate gyrus and a model of potential lineage relationship.

1.2 Adult neurogenesis and functional plasticity in neuronal circuits

The adult brain is 'a plastic place' hence flexible and easily influenced, and to ensure that the mature nervous system's control of behaviour is flexible in the face of a varying environment, morphological and physiological changes are possible at many levels, including that of the entire cell. In two areas of the adult brain, the olfactory bulb and the dentate gyrus, new neurons are generated throughout life and form an integral part of the normal functional circuitry. This process is not fixed, and is highly modulated, revealing a plastic mechanism by which the brain's performance can be optimized for a given environment. The functional benefits of this neural plasticity, however, remain a matter for debate as advocated by Pierre-Marie Lledo et al (2006).

From reports addressing specific stages of hippocampal adult neurogenesis, several principles exist which tend to describe events from the basic characterization of the process, to more complex processes and the modulators associated with the process. Neuronal developmental milestones have been reported to be highly conserved within the confines of embryonic, early postnatal and adult neurogenesis.

In the embryonic development, juvenile neurons receive GABAergic synaptic inputs before the formation of glutamatergic inputs, and depolarized by GABA due to increased expressions of the chloride importer NKCC1 at this stage (Ge et al., 2008). In adults, the process is significantly slower with regards to rate of neuronal maturation when compared to embryonic development (Overstreet-Wadiche et al., 2006a; Zhao et al., 2006). The functional significance of a prolonged development in adult neurogenesis remains unknown, and accelerating maturation rates occasionally leads to aberrant integration of these newborn neurons in the dentate gyrus of the hippocampusas reported in literature (Duan et al., 2007; Overstreet-Wadiche et al., 2006b; Parent et al., 1997).

In addition, precursor subtypes display significant plasticity in their lineage choice. An example reported in literature seen in olfactory bulb neurogenesis where about 30,000 new neurones are added / day in adult mice, more than 97 % of these differentiate into granule cells and the remaining become periglomerular neurones (Lois and Alvarez-Buylla. 1994, Winner et al. 2002). DCX positive neuroblasts in the adult SVZ can be converted to oligodendrocyte fate upon demyelination of the corpus callosum (Jablonska et al., 2010), while retroviral-mediated Mash1/Ascl1 expression redirects neurogenic intermediate progenitors into an exclusive oligodendocyte lineage in the adult SGZ (Jessberger et al., 2008). Similar critical periods exist for specific aspects of adult neurogenesis, in both SGZ and SVZ. Therefore, neural progeny survival exhibits two critical periods; one at the intermediate progenitor and neuroblast stage (Platel et al., 2010; Sierra et al., 2010) and the other at the immature neuron integration stage (Mouret et al., 2008; Tashiro et al., 2006). Research has also shown that newborn neurons exhibit enhanced synaptic plasticity of their glutamatergic inputs within a critical period (Ge et al., 2007; Nissant et al., 2009; Schmidt-Hieber et al., 2004). There are two possible functional consequences of the time-dependent facilitation for associative plasticity of adult-born neurons, such that enhanced plasticity may give adult-born neurons an advantage in the competition with mature neurons for a selective formation and stabilization of afferent and efferent synaptic connections (Tashiro et al., 2006; Toni et al., 2007). This may also allow integrated adult-born neurons to make unique contributions to information processing during this period.

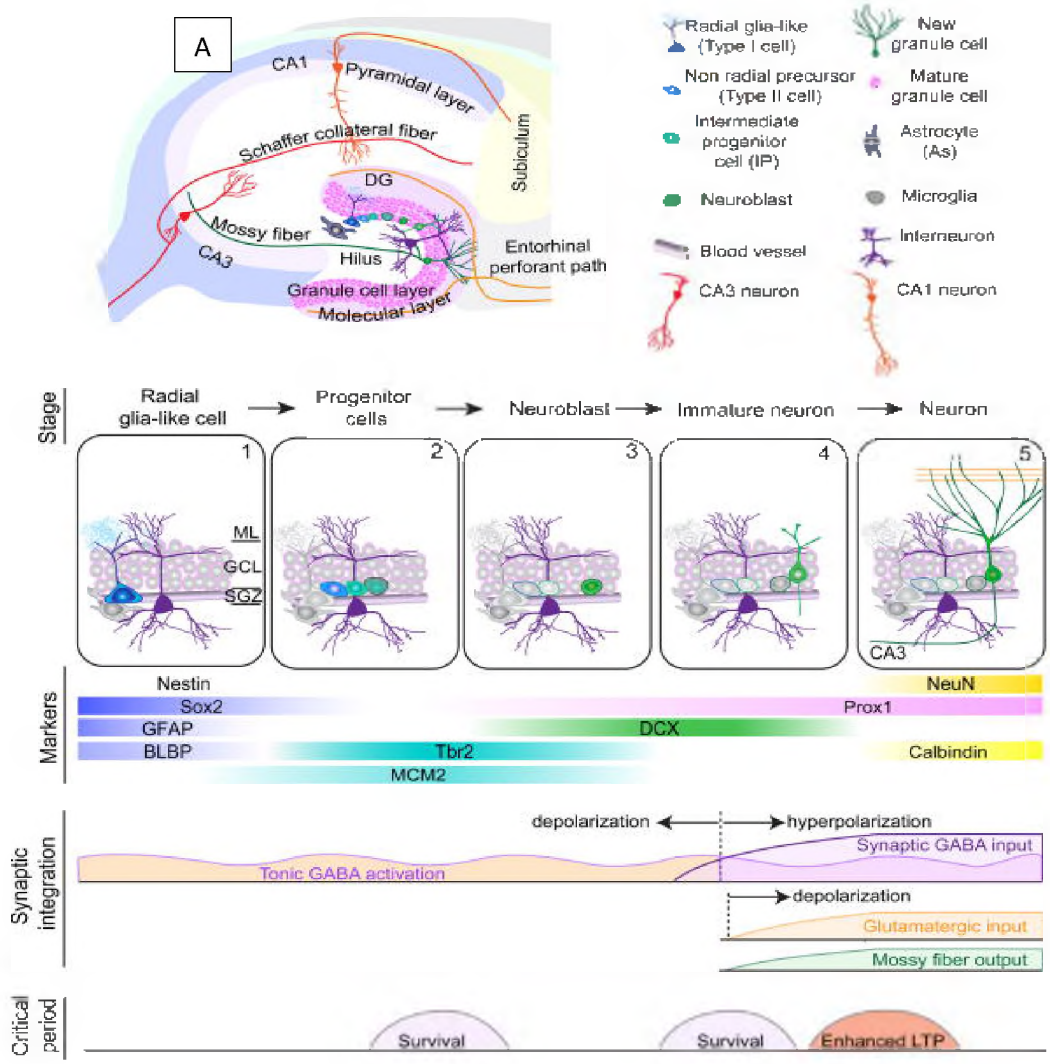


Figure 1.2: Adult neurogenesis in the dentate gyrus of the hippocampus. Ming and Song (2011)

Summary of the five developmental stages during adult hippocampal neurogenesis: (1) activation of quiescent radial glia-like cell in the subgranular zone (SGZ); (2) proliferation of non-radial precursors and their intermediate progenitors; (3) generation of neuroblasts; (4) integration of immature neurons; (5) maturation of adult-born dentate granule cells. The expression of stage-specific markers, sequential process of synaptic integration, and the critical periods regulating their survival and plasticity are also shown above. ML: molecular layer; GCL: granule cell layer; SGZ: subgranular zone; GFAP: glial fibrillary acidic protein; BLBP: brain lipid-binding protein; DCX: doublecortin; NeuN: neuronal nuclei; LTP: long-term potentiation.

1.3 Functional significance and integration in adult neurogenesis

Considering the functional significance of adult neurogenesis, the common perception is that new neurons in the adult brain would be beneficial to overall function. Reports show that during evolution, adult neurogenesis decreased with increasing brain complexity (Kempermann et al 2004). Lower vertebrates such as lizards, can regenerate entire brain and body parts, while neurogenesis in adult mammals is restricted to a few brain regions. Hence there should be a trade-off between the benefits accrued from new neurons and problems caused for the network into which they integrate. In the two neurogenic regions of the adult mammalian brain, hippocampus and SVZ/olfactory system, the benefits outweigh the 'problems', as the 'function' of the new neurons becomes a central issue. It is someworth self-evident that a neuron is not considered a true neuron unless it functions as one, hence a major challenge remains to define the concept of what 'functionality' actually means, and how to measure it.

'Function' in adult neurogenesis can be considered at various levels, 1-cellular, 2-network, and 3-systemic levels. For the purpose of this research, we restrict this to the network level as neuronal function is basically communication (dendrites and synapse formation). Adult neurogenesis is a complex multi-step process originating from precursor cells (stem cells and lineage determined progenitor cells) in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Lois and Alvarez-Buylla, 1994). In both systems, the stem cells appear to have astrocytic properties (Doetsch et al 1999, Seri et al 2001). In the dentate gyrus of the hippocampus, these cells designated type-1 cells or B cells (Doetsch et al 1999, Seri et al 2001) show the electrophysiological features of astrocytes: passive membrane properties and potassium currents (Flippov et al 2003, Fukuda et al 2003). By contrast, type-2 cells are highly proliferative progenitor cells in the same region, having electrophysiological properties originally described for glial precursor cells. Nonetheless, they appear to be in the neuronal lineage, as early neuronal markers and sodium currents (basic electrophysiological

hallmarks of neurons) can be found in some type-2 cells. Activity-dependent regulation of neurogenesis as seen, following exposure to environmental complexity, also affects these type-2 progenitor cells (Kronenberg et al 2003). Therefore, the first indication of 'neuronal function' appears at the level of the precursor cells (Kempermann et al 2004).

The stability of new neurons seems to be an important prerequisite for function, although from a purely theoretical view, adult neurogenesis could be transient and yet functional. In fact, new neurons survive for a long time in both the hippocampus and the olfactory systems (Winner et al 2002, Kempermann et al 2003). The decision for terminal neuronal differentiation is made early in development: once immature neurons are about one week into the post-mitotic stage, they are likely to persist (Kempermann et al 2003). Functional analysis of single cells becomes particularly important when aiming to grow neurons of desired neurotransmitter types in culture. Song et al (2002) found that co-culturing adult-derived precursor cells with astrocytes induced their development into electrically active neurons that formed networks *in vitro*. Organo-typic slice cultures and co-culture systems can add another level of complexity to *in vitro* studies and blur the border of the network level. Benninger et al (2009) showed that neurons derived from embryonic stem cells *in vitro* functionally integrated into early postnatal hippocampal slice cultures, and the implanted cells gradually expressed a mature profile of receptors with voltage gated channels that became synaptically integrated.

Functional integration on a network level *in vivo* requires that the new neurons extend dendrites and axons and form synapses. As early as 1988, Stanfield et al (1988) demonstrated that new granule cells in the adult dentate gyrus extend axons along the mossy fiber tract. This structural integration was later confirmed by two other reports (Hastings et al., 1999, Markakis et al., 1999). It is not clear how predictive the structural integration is for actual functionality, but it is an obvious requirement. Wichterle et al (2002) recapitulated motor neuron differentiation from embryonic stem cells in vitro and showed that after implantation the new cells formed adequate

synapses on the target muscles. Consequently, 'function' is an elusive term with a variety of theoretical and practical meanings on different conceptual levels.

Although adult neurogenesis has so far, been addressed on the levels of cells, networks and systems, functional significance of new neurons has also been inferred on a fourth level, the cognitive level. This is at the level of the individual, with its psychological and social contexts. Such implications are important but difficult and sometimes problematic, as these conceptual levels overlap. Neurons use synapses to form networks and integration of networks leads to the establishment of complex circuitry in functional systems (the hippocampus or the olfactory system). Tests of function are very different at different conceptual levels. Thus, 'function' will mean very different things depending on the experimental situation (Kempermann et al 2004).

After labelling newly generated hippocampal cells with a retrovirus expressing a reporter gene, van Praag et al (2002) showed that, over weeks, new cells developed electrophysiological characteristics similar to older granule cells. Thus, adult-generated neurons became electro-physiologically functional *in vivo* and were integrated as granule cells. In the olfactory bulb, two types of neurons are generated during adulthood. Most neurones develop into granule cells in the glomeruli, but a small percentage (5%) become interneurons in the periglomerular layers (Winner et al 2002). Carleton et al (2003) followed retroviral labelled neuroblasts from the SVZ to the olfactory bulb, and identified five electro-physiologically distinguishable stages of neuronal maturation which are activation of cells, proliferation and transient amplifying of cells, generation of neuroblasts, chain migration of neuroblasts and synaptic integration of granule cells. These neuroblasts were reported to travel through the rostral migratory stream where they radially migrate into the granule cell layer (Lois and Alvarez-Buylla, 1994). Spontaneous synaptic activity is seen late and spiking activity much later, after the cells had completed migration to their target areas. This delayed maturation might be protective to both the migrating cells and the networks into which they integrate. Very rapidly after entering the target region, the new cells express g-

aminobutyric acid (GABA) and glutamate receptors and become responsive to stimuli from the olfactory nerve layer of the bulb (Belluzi et al 2003). Carle´n et al (2002) infected entorhinal cortex neurons with a pseudorabies virus, which was propagated trans-synaptically and found reporter gene expression in new neurons of the dentate gyrus and the CA3 region, suggesting that the new granule cells became part of the synaptic network. In the olfactory bulb, olfactory stimuli evoked an upregulation of c-fos gene expression in newborn neurons, which also supports the theory of functional synaptic integration. Jessberger et al (2003) used activity-dependent gene regulation in the hippocampus to show that training a hippocampus-dependent learning task (Morris water maze), induced c-fos expression in newborn granule cells. Over time in weeks, 80% of the new neurons matured and were responsive to general synaptic stimulus (systemic application of kainic acid), allowing a first glimpse at functional maturation across the entire population of new cells (Kemperman et al 2004).

1.4 Potential relevance of Adult Neurogenesis

In adult brain studies, the dorsal (septal pole) and ventral (temporal pole) hippocampi are implicated in learning/memory and also affective behaviours respectively, whereas the olfactory bulb is involved in olfaction. Following initial discovery of neurogenesis in the postnatal rat hippocampus, Altman proposed that new neurons are critical for learning and memory (Altman, 1967). With more research, analyses at the cellular, circuitry, system and behavioral levels over time, have further generated evidence supporting critical contributions of adult-born neurons to hippocampal and olfactory bulb functionality (Deng et al., 2010; Lazarini and Lledo, 2011); Gage et al., 2011; Hen et al., 2011).

At cellular levels, newborn neurons display properties that are distinct from mature counter parts. Synaptically-connected newborn neurons exhibit hyper-excitability with enhanced synaptic

plasticity of their glutamatergic inputs during a critical period of maturation in both hippocampus and olfactory bulb (Ming and Song 2011), which may allow newly integrated adult-born neurons to make unique contributions to information processing. At circuitry levels, adult-born neurons are responsible for certain special properties of the local circuitry. As shown by long-term potentiation (LTP) of evoked field potentials induced by tetanic stimulation of the afferent medial perforant pathway is abolished by radiation to abrogate adult neurogenesis (Snyder et al., 2001). *In vivo* documentations from the dentate gyrus in anesthetized mice shows that removal of adult neurogenesis leads to decreased amplitude in perforant-path evoked responses and a significant increase in both the amplitude of spontaneous γ-frequency bursts in the dentate gyrus and the synchronization of dentate neuron firing to these bursts (Lacefield et al., 2010). Howbeit the system level, a number of models of adult neurogenesis have provided clues on how addition of new neurons may alter neural network properties and have suggested distinct roles for adult-born neurons at different stages of neuronal maturation (Aimone and Gage 2011; Petreanu and Alvarez-Buylla 2002; Carleton et al. 2003).

At behavioural levels, targeted behavioural tests and sophisticated genetic approach enhance temporal and spatial specificity. Many studies have shown positive correlation between neurogenesis and performance of specific behavioural tasks. Proof came from reports using the effect of anti-mitotic agent MAM to hinder hippocampal neurogenesis and disrupt trace eye-blink conditioning and trace fear conditioning, but not contextual fear conditioning and spatial memory, all of which were contemplated to be hippocampus-dependent forms of memory (Shors et al., 2001). Moreso, irradiation in rodents and genetically modified mice studies have similarly been used to inducibly eliminate adult neurogenesis providing substantial evidence that newborn neurons in the adult brain are required for some, but not all hippocampus or olfactory bulb-dependent tasks (Deng et al., 2010; Lazarini and Lledo, 2011). Collectively, studies have indicated significant contributions of adult hippocampal neurogenesis to spatial-navigation learning and

long-term spatial memory retention, spatial pattern discrimination, trace conditioning and contextual fear conditioning, clearance of hippocampal memory traces and rearrangement of memory to extra-hippocampal substrates (Deng et al., 2010; Gage et al. 2011). The potential role of adult hippocampal neurogenesis in affective behaviour is still under debate (Gage et al. 2011and Hen et al. 2011). Moreso, olfactory bulb neurogenesis may regulate pheromone-related behaviors, such as mating and social recognition (Feierstein et al., 2010). Also, postnatal-born granule cells subjected to sensory deprivation during the critical period, display fewer synaptic spines (Saghatelyan et al. 2005). Kelsch et al. (2009) confirmed this finding using genetically encoded markers for excitatory synapses in adult-born granule cells of the olfactory bulb. Loss of input and output synapses triggered by sensory deprivation occurs only in early synaptic development and not when sensory deprivation is introduced after synaptic development has been completed. This suggests that the critical time for the survival of new neurones depends on sensory inputs coinciding with the stage when neurons have a high degree of plasticity, facilitating the prunning of synaptic organizations. Nissant et al. (2009) demonstrated that long-term potentiation can be induced in adult-born neurons during early stages of maturation, but not after this period. Hence the effects of olfactory deprivation on synaptic development are complex. Adult-born cells that survive after sensory deprivation, display increased density of proximal input synapses in their unbranched apical dendrite (Kelsch et al. 2009), suggesting that, neurons may compensate for the absence of sensory inputs, by receiving additional excitatory drive (by elevating their activity level above the threshold required for survival). Otherwise, abnormal adult neurogenesis contributes to pathophysiological states. For instance, seizure-induced SGZ neurogenesis may contribute to epileptogenesis and long-term cognitive impairment (Jessberger et al., 2007; Kron et al., 2010).

Interestingly, a small number of newborn neurons can affect global brain function. The possibility resides in the capacity of adult-born neurons acting as encoding units and as active modifiers of

mature neuron firing, synchronization and network oscillations (Ming and Song 2011; Carleton et al 2003; Kelsch et al 2008; Mizrahi 2007; Whitman and Greer 2007a). In addition, adult-born neurons are selectively activated by specific inputs as indicated by immediate early gene expression in both the hippocampus and olfactory bulb (Kee et al., 2007; Ramirez-Amaya et al., 2006; Belnoue et al., 2011). Also, adult generated neurons strongly inhibit local circuitry outputs as seen in the olfactory bulb, where granule and periglomerular neurons inhibit many principal mitral and tufted cells which can be demonstrated by fate mapping of the cells (Kelsch et al., 2007; Mori et al., 1983; Merkle et al 2007; Ming and Song 2011).

The phenomenon of 'independent micro-circutry' poses that specific populations of granule cells target only a class of neurons and exclusively form synapses (Mori 1987) and this concept is consistent with the photomap model of circuit assembly (Rakic et al, 2009), such that genetically engineered stem cells can generate specific neural profiles to replace damaged/injured or diseased ones. In the hippocampus, adult-born dentate granule cells, while making a small number of extremely potent, large mossy fiber connections with target CA3 pyramidal neurons, innervate tens of hilar basket interneurons, each of which in turn inhibits hundreds of mature granule cells in the dentate gyrus (Freund and Buzsaki, 1996).

Additionally, adult-born neurons modify the local circuitry through selective activation of modulatory pathways. Recent studies (How, when and where new inhibitory neurobes release neurotransmittersin the adult olfactory bulb; The neuropil of the periglomerular region of the olfactory bulb; The distinct temporal origin of olfactory bulb interneurone subtypes; Generation of distinct types of periglomerular olfactory bulb interneurons during development and in adult mice: implication for intrinsic properties of the subventricular zone progenitor population; Adult-generated neurons exhibit diverse developmental fates) suggest that these young neurons contact several distinct subtypes of local interneurons (Bardy et al., 2011; Pinching and Powel 1971;

Batista-Brito et al,. 2008; De Marchis et al., 2007; Whitman and Greer 2007a), thus introducing dis-inhibition and some degree of heterogeneity to their development and organisation.

In the dentate gyrus, granule cells are known to innervate hilar mossy cells, which in turn activate many mature dentate granule cells contralaterally (Ming and Song, 2011). Hence the need to understand the contribution of potential modulatory inputs of adult-born neurons to the olfactory bulb and dopaminergic inputs to the dentate gyrus (Mu et al., 2011).

Maturation sequence show that neurones develop GABAergic inputs first, others glutamatergic inputs with a continuous increase in their excitatory input frequency till about 6 weeks after birth, when the dendritic arbour are fully developed (Grubb et al. 2008). As they mature, they become more stable with functional changes occurring at restricted postsynaptic sites (Livneh et al. 2009, Murphy et al. 2004, Zou et al. 2004).

1.5 BACKGROUND OF THE STUDY

The adult mammalian brain retains neuronal stem cells which can develop into neurons or glia (Kuhn and Svendsen, 1999). Adult neurogenesis is the process of recruiting neuronal progenitor cells in the adult brain to yield new neurones, and is affected by genetic, environmental and pathologic factors. Some environmental factors reported to modulate neurogenesis in the dentate gyrus are enrichment activities, social isolation, alcohol consumption, odour deprivation, maternal separation and prenatal stress (Fabner et al 2010; Schloesser et al 2010). In pathologic conditions, neurogenesis is adversely affected by the effects of most disease processes, as noted in literature that stress, diabetes, neurologic disease, stroke and traumatic injuries negatively affect adult neurogenesis (Taupin, 2005). All three stages of adult neurogenesis: proliferation, cell survival, and cell differentiation have been observed to differ in the dentate gyrus of the following mice strains; C57BL/6 with highest neural stem cell proliferation and the BALB/c, CD1 with greater

number of cells that became neurones (Gage and Kempermann, 1999).

Adult neurogenesis is a common feature of the mammalian brain and has been established to occur at active neurogenic sites; the subventricular zone of the anterior lateral ventricular wall and the subgranular layer of the dentate gyrus of the hippocampus in approximately 70 mammalian species ranging from small sized rodents through elephants (Patzke et al. 2013). While new cells and their differentiation into neurons appear to occur across species from different environments, it is possible that integration into adult circuitry to augment functionality may differ. During integration of differentiating neurones synaptogenesis and spinogenesis occurs hence one can predict that animals in a more complex environment may have greater rates of circuit integration when compared to those of a less complex environment. To understand the mechanisms and functions of adult neurogenesis, a good knowledge of the complete process occurring in an active neurogenic site i.e. hippocampus (cell derivation from the subgranular layer, maturation of the stem cell niche and functional integration of neurons) is necessary. According to Kempermann (2004), the function of adult neurogenesis operates at cellular network systems and cognitive behavioural levels. Functional integration on a network *in vivo* requires that the new neurones extend dendrites and axons to form synapses. Kempermann theorised that the hippocampus presumably serves as "gateway to memory". New neurones are added at the bottle neck of the circuitry in response to functional stimulation implying that adult neurogenesis is not involved in memory increase per se but in the long-term adjustment of the processing unit" Hippocampus" to increase functional needs.

1.5.1 SYNAPTOGENESIS

Synaptogenesis is a process involving the formation of a neurotransmitter release site in the presynaptic neuron and a receptive field at the postsynaptic partners, and the precise alignment of pre- and post-synaptic specializations (Jin 2005). A form of synaptogenesis would be the

formation of chemical synapses, found between two neurones or a neuron and glia. Synapses are morphologically distinct sub-cellular junction structures, composed of pre-synaptic terminal, a post-synaptic target and the synaptic cleft aligning the pre- and post-synaptic specializations (Pappas and Purpura, 1972; Cowan et al., 2001). The presynaptic terminal is characterized by a cluster of synaptic vesicles surrounding an electron-dense membrane specialization; and the postsynaptic site contains densely packed ion channels and signal transduction molecules. Both the pre- and post-synaptic specialization, display variable appearance, depending on the organism and neuron type (De Camilli et al., 2001; Sorra and Harris, 2000; Zhai and Bellen, 2004). Synapse formation has been found to occur in two general patterns: *en passant*- synaptic boutons are formed along the axon shaft; or *terminaux*-synaptic boutons are formed at the end of axon branch (Jin 2005; White et al., 1986). The presynaptic assembly involves coordinated actions of several inter-dependent events (Murthy and De Camilli, 2003) including the formation of an electron-dense pre-synaptic density at the membrane apposition with the post-synaptic cell; the clustering of synaptic vesicles surrounding the density; and the establishment of an active zone with an appropriate ratio of docked vesicles to the pre-synaptic density. In the vertebrate nervous system, synaptic activity is said to play a crucial role in shaping synaptic patterns (Jin 2005).

1.5.1.1 Stages of synaptogenesis in adult neurogenesis

The three main types of neurons added to the adult brain are the granule cells of the olfactory bulb, peri glomerular neurons (PGNs) in the olfactory bulb and the granule cells in the dentate gyrus (DG) of the hippocampus. Granule cells of the olfactory bulb constitute the largest population of adult-born neurons. They are GABAergic interneurons connecting to the lateral dendrites of the principal neurons in the olfactory bulb (mitral and tufted cells). Periglomerular neurons are GABAergic and/or dopaminergic interneurons that modulate incoming information from olfactory sensory axons connecting to the apical dendrites of the principal neurons of the olfactory bulb.

Granule cells in the DG are excitatory neurons receiving inputs from the entorhinal cortex and projects to the CA3 region of the hippocampal formation (Kelsch et al, 2010). This process differs between the olfactory bulb and the dentate gyrus of the hippocampus and for the purpose of this research we focus on the dentate gyrus of the hippocampus.

1.5.1.2 Synaptogenesis in adult born neurons of the dentate gyrus

i. Stages of Synaptic Development

The third class of adult-born neurons in mammals is the granule cells of the dentate gyrus, (with the other two being granule cells of the olfactory bulb and priglomerular neurones) arising from progenitors within the subgranular zone of the dentate gyrus of the hippocampus, beneath the granular layer where mature neurons reside. Approximately, 9000 new granule cells are produced daily in the dentate gyrus of young rats (Cameron and McKay, 2001). The neurons receive main excitatory inputs from the entorhinal cortex and provide glutamatergic input primarily to the excitatory pyramidal neurons and inhibitory interneurons within the CA3 region of the hippocampus (Kelsch et al., 2010). Consequently, the dentate gyrus acts as the main entry point for entorhinal cortex inputs into the hippocampus, relaying information to CA3 for further processing before it is returned to the entorhinal cortex via CA1.

Distinct stages of neuronal maturation of adult-born granule cells of the dentate gyrus largely recapitulate those occuring during perinatal development, although at a slower pace (Overstreet-Wadiche et al., 2006a; Zhao et al., 2006). This could be due to the upregulation of DISC1 proteins in the adult dentate gyrus of the hippocampus, which slows the increase in spine density of the granule cells of the dentate gyrus during their development (Duan et al., 2007). The new granule cells of the dentate gyrus first receive GABAergic input to their dendrites at approximately one week after they are generated. This innervation is initially depolarizing until two to four weeks' post birth, when it becomes hyperpolarizing (Esposito et al., 2005), owing to the transient

expression of the inward chloride transporter sodiun-potassium-chloride-cotransporter (NKCC1) in immature neurons, hence resulting in an elevated intracellular chloride concentration when compared to mature neurons (Ge et al., 2006). The expression of this transporter is necessary for normal development to occur as its ablation has been found to lead to severely delayed neuronal maturation (Ge et al., 2006). In the second week after birth, dendrites of granule cells start to form spines and receive glutamatergic inputs, with their GABAergic inputs becoming predominantly peri somatic (Esposito et al., 2005; Ge et al., 2006; Kelsch et al., 2010). At the same time, axonal projections from new neurons reach the CA3 region and begin to form contacts that continue to mature for months (Toni et al., 2008). By two months' post birth, adult-born neurons are seen to have similar morphological and electrophysiological properties to those formed during perinatal development (Ge et al., 2007; Laplagne et al., 2006, 2007).

ii. Activity dependent Neuronal Survival

Similar to the events occurring with granule cells in the olfactory bulb, 50% of the new granule cells of the DG of the hipocampus born in the adult brain die by four weeks of age (Kempermann et al., 1997a). Their survival is most sensitive to environmental influences between the first and third weeks of their development after birth, for adult neurogenesis in both the dentate gyrus and olfactory bulb (Rochefort et al., 2002; Tashiro et al., 2006; Mandairon et al., 2006; Alonso et al., 2006; Livneh et al., 2009).

Levels of neurogenesis and subsequent survival of granule cells of the DG of the hippocampus are strongly influenced by neuronal activity. Increased levels of adult neurogenesis in the dentate gyrus accompany changes in experiences through exercise or enriched environments (Kempermann et al., 1997b; van Praag et al., 1999). Reports also show that new neurons activated during learning are preferentially selected for incorporation into active dentate gyrus circuits (Kee et al., 2007). In addition, new granule cells of the DG whose *N*-methyl-D-aspartate (NMDA)

receptor-mediated input are eliminated, experience drastic reduction in survival rates (Tashiro et al., 2006), supporting reports describing events in the olfactory bulb, where neuronal activity plays a role in selecting new neurons that would eventually survive and become integrated into the circuitry (Yamaguchi et al., 2000; Magavi et al., 2005; Yamaguchi and Mori 2005; Alonso et al. 2006; Whitman and Greer, 2007b; Kelsch et al., 2008).

iii. Activity dependent Synaptogenesis and Pathology

Functional maturation of adult-born granule cells of the DG of the hippocampus is very sensitive to changes in activity, and the strongest agitator, of synapse formation in new granule cells of the DG have been reported to be caused by seizures (Parent et al. 1997). Experimentally induced seizures were reported to, accelerate synaptic development of new neurons such that the new cells added to an epileptic brain starts receiving, GABAergic input to their dendrites at an earlier stage (before two weeks) after birth. This is significantly earlier than that of the agitated granule cells of the DG (Overstreet-Wadiche et al., 2006b).

Actively differentiating neurons are most susceptible to develop abnormal connectivity, and morphological changes are seen only in new neurons generated within days of the seizure onset, and not neurons born earlier (Jessberger et al., 2007). Some seizure-induced synaptic changes reported in animal models include increased numbers of mushroom spines (spines with characteristically large heads) and spiny, branched basal dendrites extending into the polymorphic cell layer (Jessberger et al., 2007). Seizures have also been reported to provoke migration of new cells in the dentate gyrus. The cell bodies of new born granule cells of the DG born during seizures abnormally localize within the hilum of the dentate gyrus and fire in synchrony with CA3 pyramidal neurons, suggesting a contribution to increased excitability within the hippocampus (Scharfman et al., 2000), also reported that increased excitability blocks sensory deprivation termed 'the triggered synaptic change' in surviving neurones of the olfactory bulb (Lin et al.,

2010; Ymaguchi and Mori 2005). In both the dentate gyrus and olfactory bulb, cholinergic stimulation enhances neuronal survival and causes sustained depolarisation of the granule cells (Kaneko et al., 2006; Alonso et al., 2006; Pressler et al., 2007). Other seizure-related findings are mossy fibre sprouting, axonal projection by granule cells of the DG of the hippocampus to the supragranular molecular layer. Although the resultant effects of the sprouting remain unclear, electrophysiological studies have reported that the connectivity produces either a recurrent excitatory circuit resulting in hippocampal hyper-excitability (Okazaki et al., 1995), or recurrent inhibitions by preferentially targeting inhibitory neurons in the molecular layer (Sloviter 1992).

iv. Synaptic plasticity during a critical period

Similar to reports of events of granule cells of the olfactory bulb (Lois and Alvarez-Buylla 1994; Winner et al 2002; Carleton et al 2003; Mizrahi 2007; Kelsch et al 2008), there is a critical period within which new neurons in the adult dentate gyrus display increased synaptic plasticity when compared with mature neurons as demonstrated by enhanced ability for long-term potentiation (Schmidt-Hieber et al., 2004). This enhanced synaptic plasticity lasts until the second month after birth, thereafter decreases to levels comparable to those of the surrounding mature neurons of the dentate gyrus of the hippocampus (Ge et al., 2007) and even the granule cells of the olfactory bulb (Winner et al. 2002; Petrreanu and Alvarez-Buylla 2002; Lemasson et al., 2005). In addition, low-threshold calcium spike has been reported to boost fast sodium action potentials, contributing to long-term potentiation in the critical period (Schmidt-Hieber et al. 2004).

Synaptic plasticity in simple terms refers to changes in synaptic connections, as a consequence of developmental processes (Fig. 1.3). The critical period therefore, refers to to an established time frame in which the shaping of neural networks can be carried out. This is usually due to the required changes either in the structure or function of the developing neuronal circuit. As a

replacement and developmental mechanism for adult neurogenesis, the critical period for synaptic plasticity has been found to be between four (4) and six (6) weeks after the birth of these neurones. At this stage, these cells exhibit long term potentiation and an increased potentiation amplitude with a decreased induction thresh-hold. This developmentally regulated synaptic expression of the MR2B contains NMDA receptors and the plasticity expressed, although transient provides for fundamental maintenance and stability for the mature circuitry (Ge et al., 2007)

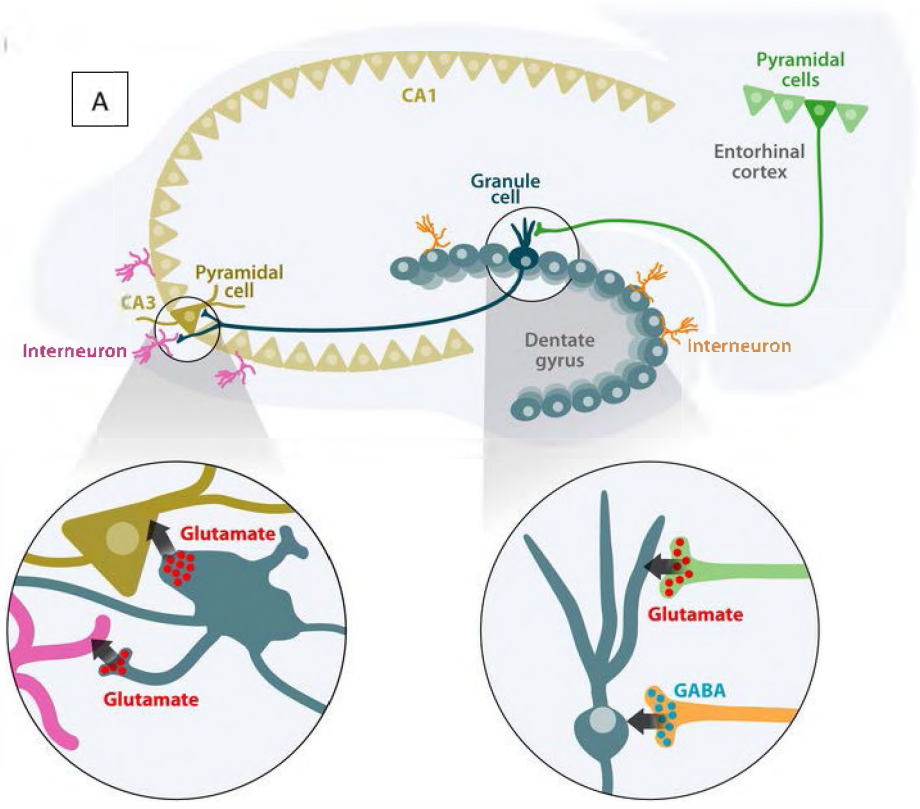


Figure 1.3: Adult neurogenesis in the dentate gyrus. Kelsch et al (2010)

Adult-born granule cells in the dentate gyrus, and their synaptic wiring with the hippocampus and other circuits. Showing synaptic organization of dentate granule cells, genetic labeling of output synapses along axon collaterals of dentate granule cells, genetic labeling of input synapses in apical dendrites of granule cells, and the dentification of inhibitory innervation on the soma of adult-born dentate granule cells.

The functional implication of adult hippocampal neurogenesis in terms of integration of new neurons in the dentate gyrus of the hippocampus with existing neural circuitry of animals from different environment has till date not been studied in detail. Kelsch et al (2010) reported that synapse formation during adult neurogenesis raises several intriguing questions; does synapse formation in adult born neurones simply recapitulate steps occurring in embryonic and neonatal development or is it regulated by specific mechanisms specialised for integration into functioning circuits? How do new neurones synapse with mature circuits without disrupting existing connectivity? Therefore, in considering functionality and integration, the impact of new neurons on adult neuronal circuitry can be determined by the neuronal survival, physiologic property and synaptic connectivity (Kelsch et al, 2010). Electrophysiologic recording and transgenic labelling provides functional information on structural observations in adult-born neurons. For instance, in the granule cells of the olfactory bulb which have been studied in details, reports show functional differences between deep and superficial granule cells, extending to neuronal survival (Imayoshi et al. 2008, Lemasson et al. 2005) supporting earlier original observation reports by Bayer (1980). The frequency and amplitude of excitatory and inhibitory synaptic inputs show changes in connectivity of new neurons during maturation and also the effects of diverse manipulations. Carleton et al. (2003) and van Praag et al. (2002) used electrophysiology to describe synaptic properties of new neurons as they matured and integrated into circuits. A significant contribution reported is the demonstration of how new neurons display enhanced synaptic plasticity when compared with fully matured neurons in both the olfactory bulb and dentate gyrus (Nissant et al. 2009, Schmidt-Hieber et al. 2004). Scharfman et al. (2000) reported that different manipulations affect neuronal integration in the adult brain differently, using the effects of seizures on synaptic properties of adult-born dentate granule neurons.

Critically the adult hippocampal granule cell layer is made up of a heterogeneous neuronal population, originating from three distinct developmental stages: late embryonic, early postnatal

and the adult brain (Altman and Bayer, 1990). Notably, these newborn dentate granule cells exhibit enhanced excitability attributable to their high input resistance and expression of low threshold T-type calcium channels (Ambrogini et al. 2004; Schmidt-Hieber et al. 2004), so that a tiny fraction of the postsynaptic current is sufficient for cells to reach firing threshold (Schmidt-Hieber et al. 2004). The T-type calcium channels in the newborn cells of the dentate gyrus are activated at a membrane potential of approximately 56 mV generating isolated low threshold calcium spikes / boost overshooting action potentials. In addition to these, the cells have a slow membrane time constant (120 milliseconds) and consequently, a slow decay time course, which results in an effective temporal summation of excitatory synaptic inputs. The expression of the calcium binding protein calbindin in newborn neurons have been reported to be lower when compared to the neighbouring mature granule cells of the dentate gyrus (Muller et al. 2005), indicating a low calcium buffering capacity. Stocca et al. (2005) reported that the decay time constant of the calcium transients, measured with high and low affinity calcium sensitive fluorescent dyes, were relatively slow, and likely attributable to low expression of calcium pumps and sodium/calcium exchangers leading to an effective temporal summation of calcium signals. These were reported to be important for the calcium dependent regulation of fibre outgrowth and synaptic plasticity of these cells.

Newborn granule cells in adults indeed exhibit unique properties of synaptic plasticity (Schmidt-Hieber et al. 2004). Schmidt-Heiber et al (2004) reported that during spatial exploration and learning tasks, the entorhinal input fibres fire action potentials modulated by theta frequency (4–8 Hz). The induction of long-term potentiation in granule cells of the dentate gyrus investigated by pairing short bursts of evoked excitatory post synaptic potentials with small somatic current injections repeated at 5 Hz, showed that newly generated granule cells of the dentate gyrus in the adult brain needed only to fire a single action potential to effectively induce long term potentiation, while the mature dentate gyrus granule cells needed a short burst of 5–10action

potentials during each theta cycle (Schmidt-Hieber et al. 2004). Furthermore, paring excitatory post synaptic potentials with subthreshold calcium spikes led to rapid long-term depression of the synaptic potentials in young neurons but not the mature dentate gyrus granule cells (Bischofberger et al. 2004). Hence, newly generated dentate gyrus neurones appear to have a lower threshold for induction of long term potentiation and long term depression, resulting in an enhanced bi-directional plasticity. In anesthetized animals, *in vivo*, reports show increase in neurogenesis by voluntary exercise lowering the threshold for long term potentiation of synaptic field potentials induced by theta burst stimulation (Farmer et al. 2004). On the other hand, blockade of adult neurogenesis by irradiation increased the threshold for long term potentiation induction (Snyder et al. 2001). Although voluntary exercise and irradiation might have consequences in addition to modulating neurogenesis, all of these data support that synaptic inputs to new dentate gyrus neurones have a lower threshold for synaptic plasticity. Hence newly generated granule cells in the dentate gyrus of the adult brain, exhibit enhanced excitability and synaptic plasticity as long as they express polysialylated neural cell adhesion molecule (PSA-NCAM) (Schmidt-Hieber et al. 2004).

An understanding of synaptogenesis in the adult brain of rodents from varied environments may shed more light on the putative functions of adult neurogenesis in information processing and storage, leading to new insights in the development of strategies for successful neuronal replacement therapies to identify and treat brain injuries and neurodegenerative disorders (Kelsch et al 2010).

1.5.2 SPINOGENESIS

This refers to the process of spine formation. In adult animals, young dendritic spines in contact with axons do mature at the same time as their synaptic contacts (Knott et al, 2006). The structure

of the neuronal dendritic tree, is markedly specialised among different cortical areas in the adult brain (Jacobs and Scheibel, 2002; Elston, 2003). Current data in primates suggest that there may be different temporal profiles for spinogenesis and pruning in different regions of the cerebral cortex (Huttenlocher and Dabholkar, 1997) and that these profiles may vary among species (Rakic et al., 1986). In primates, Elston et al (2009) revealed that the process of pyramidal cell synaptic refinement differs not only according to time but also location.

Adult neurogenesis has been found to be a representative of a complete recapitulation of embryonic neuronal development in the mature brain, from the fate specification of neural progenitors, migration and axon/dendritic targeting, to synaptogenesis of the newborn neurons (Ming and Song, 2005).

In the adult dentate gyrus, the proliferating neural progenitors which are located in the sub granular zone of the hippocampus give rise to new neurons, which migrate a short distance into the inner granule cell layer to become dentate granule cells. Within 4–10 days, these new cells project their axons toward the CA3 region to contact pyramidal neurons and hilar interneurons (Hastings and Gould, 1999). The dendrites that extend toward the molecular layer and continue to develop for several weeks (van Praag et al, 2002). Reports show that the first synaptic inputs are GABAergic inputs, reaches the newborn cells at the stage of the nestin- nonradial precursor cells (type-2 cells) (Wang et al., 2005). These cells maintain proliferative activity during the next stage of development and are able to express doublecortin and PSA-NCAM (Kempermann et al., 2004). In adult proopiomelanocortin (POMC)-enhanced growth factor protein (enhanced green fluorescent protein-EGFP) mice, labelled dentate gyrus cells that express PSA-NCAM also receive GABAergic synaptic inputs first (Overstreet Wadiche et al., 2004 and 2005a). Glutamatergic synaptic transmissions have also been detected in some PSA-NCAM dentate gyrus cells after stimulation of their perforant pathway (Schmidt-Hieber et al., 2004).

Dendritic spines, which are the major site of excitatory synaptic transmission, start to appear in new granule cells of the dentate gyrus at 2–3 weeks after the birth of the new born neurones/cells (Zhao et al., 2004). From these reports, there is an initial peak of spine growth, which then slows down, reaching a plateau at about 8 weeks after birth. Although the density of the mushroom spines continues to increase in size, this indicates a prolonged structural modification of the adult born granule cells of the dentate gyrus. Furthermore, timing of functional maturation may be subject to modulation. After pilocarpine-induced seizures, EGFP-labelled dentate granule cells in the adult POMC-EGFP mice exhibited elongated dendrites with functional glutamatergic synaptic inputs, suggesting that activity accelerates the functional integration of new neurons in the adult brain (Bromberg et al., 2004; Overstreet Wadiche et al., 2005b).

The hypothesis therefore is that there might be species differences in the spinous and synaptic organisation of adult born neurones of the dentate gyrus of the hippocampus. Spinogenesis and synaptic pruning during development are widely believed to sub-serve connectional specificity in the mature central nervous system through Hebbian-type reinforcement. Regional differences seen in the number of dendritic spines (putative excitatory inputs) of basal dendrites in pyramidal cells reported during spinogenesis and pruning, and in the adult, provide further evidence in support of the theory relating neural complexity and functional hierarchies in the brain (Jacobs and Scheibel.,2002; Elston, 2007; and Spruston, 2008). The stability, plasticity and diversity of spines in neural circuits in the adult nervous system are of central importance for understanding brain function as spines of different populations are said to behave differently (Yutse and Bonhoeffer 2004).

Previously, a lot of research on neuronal proliferation and maturation have been carried out but not on the nature and integration of these neurones. Hence, the latter, a very important aspect of adult neurogenesis needs to be studied in detail. In the studies for proliferation and maturation, Ki-67 (cell proliferation marker) and DCX (immature neurone marker) were respectively employed.

The integration process of new neurones which provides a functional contact needs attention. The processes of adult hippocampal neurogenesis have been seen to occur in over 70 mammalian species already studied and reported (Patzke et al., 2013a, b). For full integration to be understood, structural remodelling usually occurs at different stages; changes in length of dendritic branches, complexity of dendritic arbour, density of dendritic spine and morphology of dendritic spines (Fu and Zou, 2011).

1.6 Factors that regulate Adult Neurogenesis

Adult neurogenesis has a very high sensitivity to physiological and pathological stimuli at almost every stage of the process, from proliferation of neural precursors, to development, maturation, integration and survival of the newborn neurons (Zhao et al., 2008). Various researchers have demonstrated some impact of these factors as mentioned (Ming and Song, 2005; Zhao et al., 2008).

Adult neurogenesis is dynamically regulated by many physiologic stimuli, as exemplified by the adult SGZ, where physical exercise increases cell proliferation (van Praag et al., 1999), while an enriched environment promotes new neuron survival (Kempermann et al., 1997). In contrast, aging leads to a significant reduction in cell proliferation in both adult SGZ and SVZ (Rossi et al., 2008). Learning modulates adult neurogenesis in a complex, but specific pattern (Zhao et al., 2008). Adult SGZ neurogenesis is only influenced by learning tasks that depend on the hippocampal activity. Such that subjecting animals to specific learning paradigms mostly regulates the survival of new neurons, and the effects depend on the timing of cell birth and learning phase, which can be either positive or negative (Drapeau et al., 2007; Mouret et al., 2008).

Adult neurogenesis is also influenced bi-directionally by pathological states which are sometimes due to environmental exposure to certain toxic substances. Seizures increase cell proliferation in

both SGZ and SVZ (Jessberger and Parent, 2007). In the adult SGZ, seizures can lead to mis-migration of newborn neurons to the hilus, aberrant dendritic growth, and recurrent connections of the mossy fiber (Kron et al., 2010; Parent et al., 1997), and also, altered electrophysiological properties of GABAergic and glutamatergic synaptic inputs for newborn granule cells (Jakubs et al., 2006). Strikingly, a transient seizure induced by pilocarpine (in hours) (Parent et al., 1997) or electro-convulsion (in minutes) (Ma et al., 2009), leads to sustained increases in precursor proliferation for days and weeks, indicating a form of memory in regulation of neurogenesis by neuronal activity. In addition, focal or global ischemia improves cell proliferation and survival (Lindvall and Kokaia, 2007). Strokes have been found to induce cell proliferation and migration of newborn neurons to infarct sites, although vast majority fail to survive over long-term, presumably due to a lack of functional connections and trophic support (Arvidsson et al., 2002). Inversely, various degrees of chronic stress lead to decreased cell proliferation in the adult SGZ, while the effect of acute stress on cell proliferation and the survival of new neurons depends on paradigm, species and sex of animals (Mirescu and Gould, 2006). During neurodegeneration, activation of resident microglia, astrocytes and infiltrating peripheral macrophages release a plethora of cytokines, chemokines, neurotransmitters, and reactive oxygen species, which in turn affect various aspects of adult neurogenesis from stressors. For example, animal models of Alzheimer's disease, show that aberrant GABA signalling affects fate specification of neural progenitors and dendritic growth of newborn neurons in the aged SGZ (Li et al., 2009; Sun et al., 2009). A major negative regulator of adult neurogenesis is inflammation, induced by injuries, degenerative neurological diseases and irradiation (Carpentier and Palmer, 2009). In these cases, inflammation induced by irradiation not only diminishes the proliferative capacity and neuronal fate commitment of neural progenitors in the adult SGZ, but also disrupts the local niche with aberrant angiogenesis and increasing number of reactivated microglia cells, resulting in sustained inhibition of neurogenesis from both endogenous and transplanted neural progenitors (Monje et al., 2003).

It is clear that every single phase of adult neurogenesis can be regulated by different stimuli and each stimulus can have multiple targets. Furthermore, different stimuli interact with each other and impact the final outcome on adult neurogenesis. In general, regulation of adult neurogenesis by external stimuli is complex and the effect depends on timing, dose/duration, specificity of the paradigm, animal model (age, sex, and genetic background) and methods of analysis. These mechanistic studies may ultimately lead to new therapeutic strategies to enhance functional neurogenesis for regenerative medicine. Observations from results of the complex environment support the fact that certain environmental stimuli can cue proliferating neurones to become more adapted for more complex functions with a marked improvement in the rate of proliferation, which can help to mask early stages of neurodegenerative disease and possibly slow overt progression in the course of a neurodegenerative disease.

1.7 Environmental diversity and adult neurogenesis: Each environment is unique to its structure and life forms and can be found to influence the biological existence within them.

1.7.1 The need to study animal species and natural environments

Wiskott et al., (2006) assumed that when animals are in a stable environment, mature neurones are stable and provide needed service, when introduced into a new environment they need to learn new information in order to fit in without interfering with previously learned information. Neurogenesis may be necessary for interference avoidance hence the rodent species proposed for this research differs significantly between environments. Genetics influences rate of cell proliferation and integration. Lindsey and Tropepe (2006) and Gould (2007) gave a comparative picture of adult neurogenesis in various taxonomic groups. This research will try to observe effects of various environments on dendritic spines and synapses, as environmental enrichment exerts great effects on the brain physiology and is widely used both as an experimental and therapeutic tool (Fabner et al., 2010; Schloesser et al., 2010).

In order to compare the extent of the integration of the new neurons, it is desirable to use an animal model that can be able to provide the desired results. Thus considering an animal model having natural genes that would be able to provide the extent of changes envisaged for adult hippocampal neurogenesis, as observed in wild rodents of natural habitats. To achieve the aim of this research, we used of the Long-Evans rat as its genes comprise natural wild (male) and laboratory (female) combination.

1.7.2 Activity dependent regulation of adult neurogenesis

A major modulator / regulator of adult neurogenesis is activity and experience, as research has shown that both exercise and environmental enrichment, encourage varying degrees of activity and thus improve adult neurogenesis (van Praag et al., 1999). The molecular mechanisms underlying activity-dependent regulation of adult neurogenesis are getting clearer and they include involvement of neurotransmitters, neurotrophins, growth factors and epigenetic regulators. In the adult SVZ, the GABA released from neuroblasts promotes their migration while inhibiting precursor proliferation (Liu et al., 2005). In the adult SGZ, GABA promotes dendritic growth, synapse formation and survival of newborn neurons through CREB signalling (Jagasia et al., 2009). Also, NMDAR signalling regulates survival of neuroblasts in the adult SVZ (Platel et al., 2010) and immature neurons in the adult SGZ (Tashiro et al., 2006). Furthermore, NR2B is necessary for enhanced synaptic plasticity of newborn dentate granule cells during the critical period (Snyder et al., 2001; Gage et al., 2001; Ge et al., 2007; Ming and Song, 2011). In addition, Gadd45b and TET1, two epigenetic regulators of active DNA demethylation, promote BDNF and FGF1 expression in mature dentate granule cells in response to neuronal activation and the deletion of Gadd45b reduces activity-induced proliferation of neural progenitors and dendritic growth of newborn neurons in the adult hippocampus (Guo et al., 2011a; Ma et al., 2009).

We need an understanding of how extrinsic niche signalling is coupled to the intrinsic machinery. A better understanding of molecular mechanisms regulating adult neurogenesis will shed a lot of light on this and will require systematic analysis of putative adult neural stem cells with their progeny at different developmental stages, including transcriptome, proteome, epigenetic status, and metabolic states. A comparative approach between events in the SVZ and SGZ neurogenesis will be particularly instrumental to understand general mechanisms regulating adult neural precursors, neuronal fate commitment, subtype differentiation, development and integration in the adult brain in a further research endeavour.

1.8 Environmental enrichment and adult neurogenesis

Typical environmental enrichment paradigms are multifactorial incorporating elements of physical exercise, environmental complexity, social interactions and stress to study changes in AHN (Gregoire et al., 2014). The surrounding environment affects the physical and functional properties of the brain and early studies showed that rodents exposed to environmental enrichment consisting of large cages, social enrichment and diverse multisensory stimulation displayed increased brain sizes, altered neurotransmitter levels and behavioural changes (Rosenzweig et al., 1962; La Torre 1968; Bennett et al., 1969; Manosevitz and Joel, 1973). Diverse environmental enrichment paradigms are used, with little understanding on how individual enrichment variables such as physical exercise, cognitive stimulation, stress, and social interactions impact on specific downstream biological mechanisms as the changes in dendritic growth (Leggio et al., 2005; Fiala et al., 1978; Volkmar and Greenough 1972), synaptic plasticity (Liu et al., 2012) and adult neurogenesis (Kempermann et al., 1997).

Knowing that environmental cues influence adult neurogenesis in captive animal models, studies into the possible implications on functional integration of these newly generated neurones from wild caught species, levels of neurogenesis and subsequent survival of the granule cells of DG

becomes important. Environmental influence, animal behaviour and social interactions are combined forces that act on brain plasticity and ultimately adult neurogenesis. Therefore, we will attempt to address the question of distinguishing the stages of synaptic and spine development in fixed slices using structural features from single and double labelling immunofluorescence and relating our findings to environmental complexity.

This research will incorporate enrichment paradigms of running exercise, complex laboratory environment and natural complex environment, in an to attempt bridge the gap between spinogenesis, synaptogenesis and adult neurogenesis. Immunohistochemical techniques to investigate immature DCX immune-positive neurones have been reported (Patzke et al., 2013a and b), synaptic and dendritic spinous process identification based on neuron structural specialisation by double labelling immunofluorescence techniques will be employed. We therefore aim to investigate whether environment of wild caught animals leads to a significant difference in the rate and extent of adult hippocampal neurogenesis when compared to the laboratory environments, and also spine and synapse formation in the immature neurones within the dentate gyrus of the hippocampus of laboratory rodents exposed to enrichment paradigms.

1.9 Aims of the study

1.9.1 BROAD AIM

To investigate synaptogenesis and spinogenesis in adult hippocampal neurogenesis in the Long-Evans rats exposed to standard laboratory, exercise and complex enriched environments.

1.9.2 SPECIFIC AIMS

1. To determine the effects of environmental enrichment between the standard laboratory environment, exercise and complex enriched environment on the brain weight, body weight and the brain/body weight index in the Long-Evans rat.

2. To determine effects of environmental enrichment between the standard laboratory environment, the exercise and the complex enriched environment on neuronal proliferation (Ki-67), differentiation and the morphology of dendrite protrusions of the new neurons (DCX) in the dentate gyrus of the Long-Evans rat.

3. To determine differences in synaptogenesis between the standard laboratory environment, the exercise and the complex enriched environment in the dentate gyrus of the hippocampus of the Long-Evans rats.

4. To determine differences in spinogenesis between the standard laboratory environment, the exercise and complex enriched environment in the dentate gyrus of the hippocampus of the Long-Evans rat.

5. To determine cell numbers (Ki-67 immunopositive, DCX immunopositive and apoptotic cells) between rodents exposed to the standard laboratory environment, the exercise and complex enriched environment in the dentate gyrus of the hippocampus of the Long-Evans rat.

6. To determine differences in the volume of the dentate gyrus between rodents exposed to standard laboratory environment, the exercise and complex enriched environment.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Experimental Animals: The experimental animals used were the Long-Evan rats. In general, rodents have been shown to be good animal models for evidence of adult hippocampal neurogenesis. Eighteen rodents used for the research were sourced through the Wits Central Animal Services (CAS) and allowed to acclimatise for three weeks to the CAS environment.

The Long-Evans model is an outbred rat developed in 1915 by Dr Long and Dr Evans from cross breeding female albino Wistar Institute rats with wild males captured near Berkeley via offspring selection. The Long–Evans rat is small, resistant to oncogenesis and widely used in behavioural (learning, stress, locomotion, circadian rhythm, aggressiveness), ageing, metabolism, nutrition and toxicology (drug addiction, alcohol) studies. By 9 weeks the male attain mean weight of 300 g and can be considered fully grown. Presently, there are neither computer models nor cell cultures for investigating the process of adult hippocampal neurogenesis because this is considered a complex biological process.

The Long-Evans rat is one of the laboratory rodents, reported to perform well in an enriched environment (running exercise as single influencing factor and also in a complex enriched laboratory environment) which makes it ideal for the purpose of comparing if possible, adult hippocampal neurogenesis in rodents of wild and complex natural environments.

2.2 Age, sex and weight of experimental animals

All animals were treated and used in accordance with the University of the Witwatersrand Animal Ethics and Screening Committee guidelines (AESC Clearance No: 2014/55/B). The Long-Evans rats used for the research were adult males, to exclude the bias of hormonal influence on the female biologic and physiologic milieu. The rats weighted between 150 g and 250 g at commencement of the experiment, reaching 380 g and 500 g upon termination at sacrifice. Simple

random sampling technique was employed as animals were randomly assigned to experimental groups.

2.2.1 Housing Conditions: Animals were housed in rat cages of varied sizes; 41 cm x 25 cm x 15 cm, 41 cm x 25 cm x 15 cm with a wheel radius of 17 cm and circumference of 170 cm attached to its free end and 50 cm x 50 cm x 100 cm in a temperature controlled room with food and water supplied *ad libitum*. Handling time was to the nearest minimum as they are docile, and was limited to weekly weighing of the rats and cage cleaning once a week. The animals were randomly assigned to three groups namely Control/Standard, running wheel (exercise) and Complex enrichment created with coloured balls, cubes and tunnels for 28 days.

The choice of 28 days' exposure after 3 weeks acclimatisation of rodents to the CAS facility was made as the standard cycle duration for neurogenesis is 21 days. This ensures that previously developed neurones outside the CAS facility matures and gets phased out so that analysed new neurones, synapses and spines are actually those that commenced proliferation stage in the CAS facility to ensure the accuracy of exposure findings with regards to the context of this research in comparison with the environmental paradigms and variation.

Table 2.1: Description of animal housing environment in the laboratory

Group	No of animals	Description of exposure
Control (Standard)	6	Normal laboratory environment using empty cages for 28 days
Exercise	6	Vertical running wheel exercise for 28 as a single influencing factor
Complex enrichment	6	Coloured balls, wooden cubes and plastic coloured tunnels to simulate an enriched and complex environment for 28 days

2.3 Perfusion and removal of brain specimens

After four weeks of exposure to the different environments, the Long-Evans rats were weighed and then euthanized with an intraperitoneal injection of phenobarbitone (2 mg/kg). All animals were perfusion-fixed transcardially, initially with a rinse of 0.9 % cold saline solution, and then by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB) solution using wide bore needles and 20 ml syringes. The brains (whole brain) were carefully removed from the skull, post-fixed for 24h in 4 % paraformaldehyde in 0.1M PB, weighed and transferred into 30 % sucrose in 0.1 M phosphate buffered saline (PBS) for 72 hours, then transferred into antifreeze and stored in the freezer. Frozen sagittal sections, at 50 μm thickness were cut using a sliding microtome (Leica). Repeated series of 9 sections were then collected free-floating into a cryo-protective solution containing sodium azide in PBS and stored at -20°C till further processing. One in five series of sections were stained with cresyl violet for anatomical orientation, another for single imunolabelling against Ki-67, DCX, Synaptophysin and Synaptobrevin for maturation patterns and differentiation. Double labelling of DCX and Synaptophysin, DCX and Synaptobrevin, Synaptophysin and synaptobrevin was done for determination of synapse and spine formation. Geimsa staining was done for determination of dead cell/ apoptotic cell count.

2.3.1 Cresyl violet for Nissl staining

Processing for general brain cytoarchitecture was done using cresyl violet. Brain sections were mounted onto 0.5 % gel coated slides and allowed to dry over 48 hours prior to staining. The slides were initially immersed in a solution containing equal quantities of alcohol and chloroform (1:1) overnight to defat the sections and improve stain penetration, and then rehydrated in alcohol by placing the slides in a series of alcohol baths of decreasing concentration 100 % for 5 minutes, 95 %, 70 % and 50 % for 2 minutes each. The slides were then placed in 1 % cresyl violet solution for 1 minute and afterwards transferred into distilled water for another 1 minute. Sections were

differentiated using 1 % acetic acid, viewed under the stereo microscope and then rinsed in water to stop the reaction. Subsequently the sections were dehydrated in graded alcohol baths of increasing concentration, for the same time as recorded above (i.e. first in 50% alcohol and lastly in 100% alcohol). The acetic acid bath step acts as the differentiation step, therefore the amount of time in which the slides were allowed was dependent on the intensity of the cresyl violet stain and was monitored under the stereomicroscope until optimal staining was achieved. Once optimal staining was achieved, dehydration was continued as stated, and after the last dehydration step (5 min in 100% alcohol) the slides were cleared in 2 changes of xylene for 5 minutes each and mounted sections were cover slipped using Depex mounting medium.

2.3.2 Single labelling immunohistochemistry for Ki-67

Ki-67 is a proliferation marker present in the nucleus during the G1 to M phases of the cell cycle (Chawana et al., 2013). Brain sections were washed 3 times for 10 minutes in 0.1 M PBS and then rinsed with TBST once for 5 minutes under gentle shaking at room temperature before being transferred into the endogenous peroxidise inhibition solution for 30 minutes. Sections were again washed 3 times for 10 minutes and then treated with a blocking buffer solution (3 % normal serum in TBST) for 120 minutes. Tissues were then transferred into primary goat-anti-mouse Ki-67 antibody (1:1000, Leica, Dako, Denmark) in TBST supplemented with 2 % BSA and 3 % normal goat serum overnight at 4 °C under gentle shaking. After 48 hours, tissues were removed from the fridge and left on the shaker to equilibrate at room temperature followed by 3 times for 10 minutes' wash in TBST under gentle shaking at room temperature. Biotinylated goat-anti-mouse secondary antibody (1:1000) in TBS supplemented with 3 % normal serum was put in the wells and sections added for 120 minutes at room temperature under gentle shaking. Sections were then washed 3 times for 10 minutes each under gentle shaking at room temperature. Thirty minutes' prior time of use, the ABC reagent in TBS (1:100, A plus B) was prepared, placed in wells 1 ml

per well and the tissue sections added for 60 minutes at room temperature under gentle shaking. Sections were then washed in TBS 3 times for 10 minutes each under gentle shaking at room temperature and then by washing in 0.1 M PB (pH 7.2) two times for 10 minutes each. Sections were then pre-incubated in DAB solution, 0.05 mg/ 100 ml in TB (pH 7.2) by using 70% of 1ml per vial for 5 min (20 minutes after preparation of DAB solution) in the dark under gentle shaking at room temperature, after which 30µl (30% of 1ml) 0.5% H_2O_2 was then added to each vial, mixed and allowed to develop under visual guidance until strong nuclear staining appeared in the sections. The reaction was stopped by washing sections in TB (pH 7.2) 3 times for 10 minutes each under gentle shaking at room temperature. The tissues were then washed in PB and mounted onto 0.5 % gelatinized slides and air dried overnight. They were dehydrated in an ascending graded series of alcohols, cleared in xylene and cover slipped with Entelan. The primary antibody was omitted in the negative control tissue sections.

2.3.3 Single labelling immunohistochemistry for DCX

DCX is a microtubule-associated phosphoprotein, which is expressed in actively dividing neuronal precursor cells and their neuronal daughter cells for up to 2 – 3 weeks. The advantage of using DCX is that no pre-handling of the animal is needed (Patzke et al., 2013a).

Brain sections of 4 series were selected per group and washed 3 times for 10 minutes in 0.1 M PBS and then rinsed with TBST once for 5 minutes under gentle shaking at room temperature before being transferred into the endogenous peroxidise inhibition solution for 30 minutes. Sections were again washed 3 times for 10 minutes and then treated with a blocking solution (3 % normal serum in TBST) for 120 minutes. Tissues were then transferred into primary goat-anti-DCX antibody (1:500, Santa Cruz, Texas) in TBST supplemented with 2 % BSA and 3 % normal rabbit serum overnight at 4 °C under gentle shaking. After 48 hours, tissues were removed from the fridge and left on the shaker to equilibrate at room temperature followed by 3 times for 10

minutes' wash in TBST under gentle shaking at room temperature. Biotinylated anti-goat secondary antibody (1:250) in TBS supplemented with 3 % normal serum was put in the wells and sections added for 120 minutes at room temperature under gentle shaking. Sections were then washed 3 times for 10 minutes each under gentle shaking at room temperature. Thirty minutes' prior time of use, the ABC reagent in TBS (1:100, A plus B) was prepared, placed in wells 1 ml per well and the tissue sections added for 60 minutes at room temperature under gentle shaking. Sections were then washed in TBS 3 times for 10 minutes each under gentle shaking at room temperature and then by washing in 0.1 M PB (pH 7.2) two times for 10 minutes each. Sections were then pre-incubated in DAB solution, 0.05 mg/ 100 ml in TB (pH 7.2) by using 70 % of 1 ml per vial for 5 min (20 minutes after preparation of DAB solution) in the dark under gentle shaking at room temperature, after which 30 µl (30 % of 1 ml) of 0.5 % H_2O_2 was then added to each vial, mixed and allowed to develop under visual guidance until strong nuclear staining appeared in the sections. The reaction was stopped by washing sections in TB (pH 7.2) 3 times for 10 minutes each under gentle shaking at room temperature. The tissues were then washed in PB and mounted onto 0.5 % gelatinized slides and air dried overnight. They were dehydrated in an ascending graded series of alcohols, 70 % for 2 hours, 90 % for 2 minutes, 100 % for 5 minutes twice, cleared in xylene and cover slipped with Entelan. The primary antibody was omitted in the negative control tissue section.

2.3.4 Single labelling immunohistochemistry for Synaptophysin

Synaptophysin is an integral membrane glycoprotein found in many types of active neurones. It is believed to form the hexamer that acts as an ion channel involved in neurotransmitter uptake, synaptophysin is only found in the membrane after stimulation of the neurone (Calhoun et al., 1996).

Brain sections were washed 3 times for 10 minutes in 0.1 M PB under gentle shaking at room

temperature before being transferred into the endogenous peroxidase inhibition solution for 30 minutes. Sections were again washed 3 times for 10 minutes and then treated with a blocking solution (3 % normal serum in PBT) for 120 minutes. Tissues were then transferred into primary mouse-anti-synaptophysin antibody (1:250, Chemicon International) in PBT supplemented with 2 % BSA and 3 % normal goat serum overnight at 4 °C under gentle shaking. After 48 hours, tissues were removed from the fridge and left on the shaker to equilibrate at room temperature followed by 3 times for 10 minutes' wash in 0.1 M PB under gentle shaking at room temperature. Biotinylated goat-anti-mouse secondary antibody (1:1000) in PB supplemented with 3 % normal serum was put in the wells and sections added for 120 minutes at room temperature under gentle shaking. Sections were then washed 3 times for 10 minutes each under gentle shaking at room temperature. Thirty minutes' prior time of use, the ABC reagent in PB (1:100, A plus B) was prepared, placed in wells 1 ml per well and the tissue sections added for 60 minutes at room temperature under gentle shaking. Sections were then washed in 0.1 M PB 3 times for 10 minutes each under gentle shaking at room temperature and then by washing in 0.1 M PB (pH 7.2) two times for 10 minutes each. Sections were then pre-incubated in DAB solution, 0.05 mg/ 100 ml in PB (pH 7.2) by using 70 % of 1 ml per vial for 5 min (20 minutes after preparation of DAB solution) in the dark under gentle shaking at room temperature, after which 30 µl (30 % of 1 ml) of 0.5 % H_2O_2 was then added to each vial, mixed and allowed to develop under visual guidance until strong nuclear staining appeared in the sections. The reaction was stopped by washing sections in 0.1 M PB (pH 7.2) 3 times for 10 minutes each under gentle shaking at room temperature. The tissues were then washed in 0.1 M PB and mounted onto 0.5% gelatinized slides and air dried overnight. They were dehydrated in an ascending graded series of alcohols 70 % for 2 hours, 90 % for 2 minutes, 100 % for 5 minutes twice, cleared in xylene and cover slipped with Entelan. The primary antibody was omitted in the negative control tissue sections.

2.3.5 Single labelling immunohistochemistry for Synaptobrevin

Synaptobrevins are small, short, very abundant integral membrane proteins of synaptic secretory vesicles with a molecular weight of 18kDa. It forms part of the vesicle-associated membrane proteins (VAMP) family, occurring in isotypes 1 and 2 localised in the membranes of synaptic vesicles. It is also one of the SNARE proteins (soluble NSF attachment protein receptors, NSF is N-ethylmalemide sensitive factor a fusion protein), a carboxy terminal trans membrane region and proline rich amino terminus together with the hydrophobic carboxy terminus contains most of the amino acid differences between the VAMP1 and VAMP2 involved in the formation of the SNARE complex (Elferink et al., 1980; Trimble et al., 1990).

Brain sections were washed 3 times for 10 minutes in 0.1 M PBS and then rinsed with TBST once for 5 minutes under gentle shaking at room temperature before being transferred into the endogenous peroxidise inhibition solution for 30 minutes. Sections were again washed 3 times for 10 minutes and then treated with a blocking solution (3 % normal serum in TBST) for 30 minutes. Tissues were then transferred into primary mouse anti synaptobrevin antibody (1:1000, Chemicon International) in TBST supplemented with 2 % BSA and 3 % normal goat serum overnight at 4 °C under gentle shaking. After 48 hours, tissues were removed from the fridge and left on the shaker to equilibrate at room temperature followed by 3 times for 10 minutes' wash in TBST under gentle shaking at room temperature. Biotinylated goat-anti-mouse secondary antibody (1:1000) in TBS supplemented with 3 % normal serum was put in the wells and sections added for 120 minutes at room temperature under gentle shaking. Sections were then washed 3 times for 10 minutes each under gentle shaking at room temperature. Thirty minutes' prior time of use, the ABC reagent in TBS (1:100, A plus B) was prepared, placed in wells 1 ml per well and the tissue sections added for 60 minutes at room temperature under gentle shaking. Sections were then washed in TBS 3 times for 10 minutes each under gentle shaking at room temperature and then by washing in 0.1 M PB (pH 7.2) two times for 10 minutes each. Sections were then pre-incubated in DAB solution,

0.05 mg/ 100 ml in TB (pH 7.2) by using 70 % of 1 ml per vial for 5 min (20 minutes after preparation of DAB solution) in the dark under gentle shaking at room temperature, after which 30 µl (30 % of 1 ml) of 0.5 % H_2O_2 was then added to each vial, mixed and allowed to develop under visual guidance until strong nuclear staining appeared in the sections. The reaction was stopped by washing sections in TB (pH 7.2) 3 times for 10 minutes each under gentle shaking at room temperature. The tissues were then washed in PB and mounted onto 0.5 % gelatinized slides and air dried overnight. They were dehydrated in an ascending graded series of alcohols 70 % for 2 hours, 90 % for 2 minutes, 100 % for 5 minutes twice, cleared in xylene and cover slipped with Entelan. The primary antibody was also omitted in the negative control tissue sections.

2.3.6 Double labelling immunohistochemistry: DCX/synaptophysin; DCX/Synaptobrevin; Synaptophysin/Synaptobrevin. The marker for proliferating immature neurones (DCX) was co-labelled with markers for immature spines (Synaptobrevin) and synapses (Synaptophysin), and marker for immature synapses (synaptophysin) was co-labeled with the marker for immature spines (synaptobrevin) to determine differentiation and maturation patterns of the proliferating and daughter cells.

2.3.6.1 Double labelling immunofluoresence for DCX/ Synaptophysin

Day 1: Free floating brain sections of the Long–Evans rat were removed from the refrigerator, and allowed to equilibrate on the shaker at room temperature. Sections were then washed in 0.1 M PB (1ml/well) under gentle shaking. Sections were then incubated for 30 minutes in endogenous peroxidase inhibition solution (Methanol 50 %, 0.1 M PB 50 % and 1.66 ml H_2O_2) under gentle shaking. Sections were then washed in 0.1 M PB 3 times in 10 minutes under gentle shaking. Sections were then treated in blocking buffer solution (0.25 % Triton X in 0.1 M PB, 3 % normal goat serum and 2 % Bovine serum albumin) for 2 hours. Sections were then incubated in primary

antibody, (DCX – polyclonal Ab; 1:1000, Santa Cruz, Texas and Synaptophysin – monoclonal Ab; 1:500, Chemicon International) for 48 hours at 4 ^{0}C for 48 hours.

Day 2; Brain sections were removed from the fridge, allowed to equilibrate on the shaker for 30 minutes and washed in 0.1 M PB 3 times in 10 minutes under gentle shaking. Then the secondary antibodies were mixed in a dark room where the remaining process was completed. Goat-anti-rabbit, Alexa Fluor 488 (1:1000) staining green was used to target the DCX and Goat-anti-rabbit, Alexa Flour 647 (1:1000) staining red, used to target the Synaptophysin. Both secondary antibodies were diluted in 0.1 M PB, 3 % Normal goat serum and 2 % BSA, 500 microlitre was placed in each well and sections incubated in the dark under gentle shaking at 4 ^{0}C overnight. Sections were then taken out and washed in 0.1 M PB for 3 times in 10 minutes under gentle shaking in dark room and mounted directly from PB solution. Making sure that sections did not completely dry out, they were cover slipped using Fluoromount and stored in the fridge wrapped with foil in a dark case. Sections were then viewed on the confocal microscope where the images were also captured.

2.3.6.2 Double labelling immunofluoresence for DCX/ Synaptobrevin

Day 1: Free floating brain sections of the Long–Evans rat were removed from the refrigerator, and allowed to equilibrate on the shaker at room temperature. Sections were then washed in 0.1M PB (1 ml/well) under gentle shaking. Sections were then incubated for 30 minutes in endogenous peroxidase inhibition solution (Methanol 50 %, 0.1 M PB 50 % and 1.66 ml H_2O_2) under gentle shaking. Sections were then washed in 0.1 M PB 3 times in 10 minutes under gentle shaking. Sections were then treated in blocking buffer solution (0.25 % Triton X in 0.1 M PB, 3 % normal goat serum and 2 % Bovine serum albumin) for 2hours. Sections were then incubated in primary antibody (DCX – polyclonal Ab; 1:1000, Santa Cruz, Texas and Synaptbrevin – monoclonal Ab; 1:1000, Chemicon International) for 48 hours at 4 ^{0}C for 48 hours.

Day 2; Brain sections were removed from the fridge, allowed to equilibrate on the shaker for 30 minutes and washed in 0.1 M PB 3 times in 10 minutes under gentle shaking. Then the secondary antibody was mixed in a dark room where the remaining process was completed. Goat-anti-rabbit, Alexa Fluor 488 (1:1000) staining green was used to target the DCX and Goat-anti-rabbit Alexa Flour 647 (1:1000) staining red, used to target the Synaptobrevin. Both secondary antibodies were diluted in 0.1 M PB, 3 % Normal goat serum and 2 % BSA. 500 microlitre was placed in each well and sections incubated in the dark under gentle shaking at 4 ^{0}C overnight. Sections were then taken out and washed in 0.1 M PB for 3 times in 10 minutes under gentle shaking in dark room and mounted directly from PB Solution. Making sure that sections did not completely dry out, they were cover slipped using Fluoromount and stored in the fridge wrapped with foil in a dark case. Sections were then viewed on the confocal microscope where the images were also captured.

2.3.6.3 Double labelling immunofluoresence for Synaptophysin/Synaptobrevin

Day 1: Free floating brain sections of the Long–Evans rat were removed from the refrigerator, and allowed to equilibrate on the shaker at room temperature. Sections were then washed in 0.1 M PB (1 ml/well) under gentle shaking. Sections were then incubated for 30 minutes in endogenous peroxidase inhibition solution (Methanol 50 %, 0.1 M PB 50 % and 1.66 ml H_2O_2) under gentle shaking. Sections were then washed in 0.1 M PB 3 times in 10 minutes under gentle shaking. Sections were then treated in blocking buffer solution (0.25 % Triton X in 0.1 M PB, 3 % normal goat serum and 2 % Bovine serum albumin) for 2 hours. Sections were then incubated in primary antibody, (Synaptophysin– monoclonal Ab; 1:500, Chemicon International and Synaptbrevin – monoclonal Ab; 1:1000, Chemicon International) for 48 hours at 4 ^{0}C for 48 hours.

Day 2; Brain sections were removed from the fridge, allowed to equilibrate on the shaker for 30 minutes and washed in 0.1 M PB 3 times in 10 minutes under gentle shaking. Then the secondary antibody was mixed in a dark room where the remaining process was completed. Goat-anti-mouse,

Alexa Fluor 000 (1:1000) staining green was used to target the Synaptobrevin and Goat anti-rabbit Alexa Flour 647 (1:1000) staining red, used to target the Synaptophysin. Both secondary antibodies were diluted in 0.1 M PB, 3 % Normal goat serum and 2 % BSA. 500 microlitre was placed in each well and sections incubated in the dark under gentle shaking at 4 ^{0}C overnight. Sections were then taken out and washed in 0.1 M PB for 3 times in 10 minutes under gentle shaking in dark room and mounted directly from PB solution. Making sure that sections did not completely dry out, they were cover slipped using Fluoromount and stored in the fridge wrapped with foil in a dark case. Sections were then viewed on the confocal microscope where the images were also captured.

2.4 Geimsa staining for pyknotic cells

Geimsa is a histologic stain used for cytomorphometric studies and it stains the nucleus of the cell darkly (deep blue) and the cytoplasm faintly (pale blue). The geimsa stained nuclei were then viewed under the microscope and assessed for pyknosis (Ben Abdallah et al., 2008). Pyknotic nuclei were counted as an indicator of cell death and multiplied by the inverse of the sampling fraction. Brain sections were mounted on 5 % gel coated slides and allowed to dry over 72 hours, and the slides placed in defat solution (absolute alcohol + chloroform, 1:1) overnight. The slides were then rehydrated using graded alcohol; 100 % for 5mins, 100 % alcohol for 5mins, 95 % for 2 mins, 70 % for 2mins and 50 % for 2 mins.

Procedure: Sections were then transferred into Giemsa working solution at room temperature for 40mins, differentiated in 1% Acetic Acid 30sec, washed in running water to stop the reaction and transferred into 93% ethanol for 30sec and finally dehydrated in ascending alcohol grades; 50% for 2mins, 70 % for 2 mins, 95% for 2 mins, 100 % for 5 mins and 100 % for 5 mins. Checked microscopically, for adequate differentiation and then placed in Xylene for 5 mins and cover slipped.

2.5 Data analysis

2.5.1 Qualitative description of data

The morphologic description of findings from immunostained sections per group, standard environment, exercise as single influencing factor group and the enriched environment will be presented from images taken on Axiovision microscope and confocal microscope.

The neurone structures seen are post mitotic neurones. In the complex enriched environment, post-mitotic neurone of category F (Plumpe et al., 2006), the exercise group and standard group were found to have post mitotic neurones of category E (Plumpe et al., 2006).

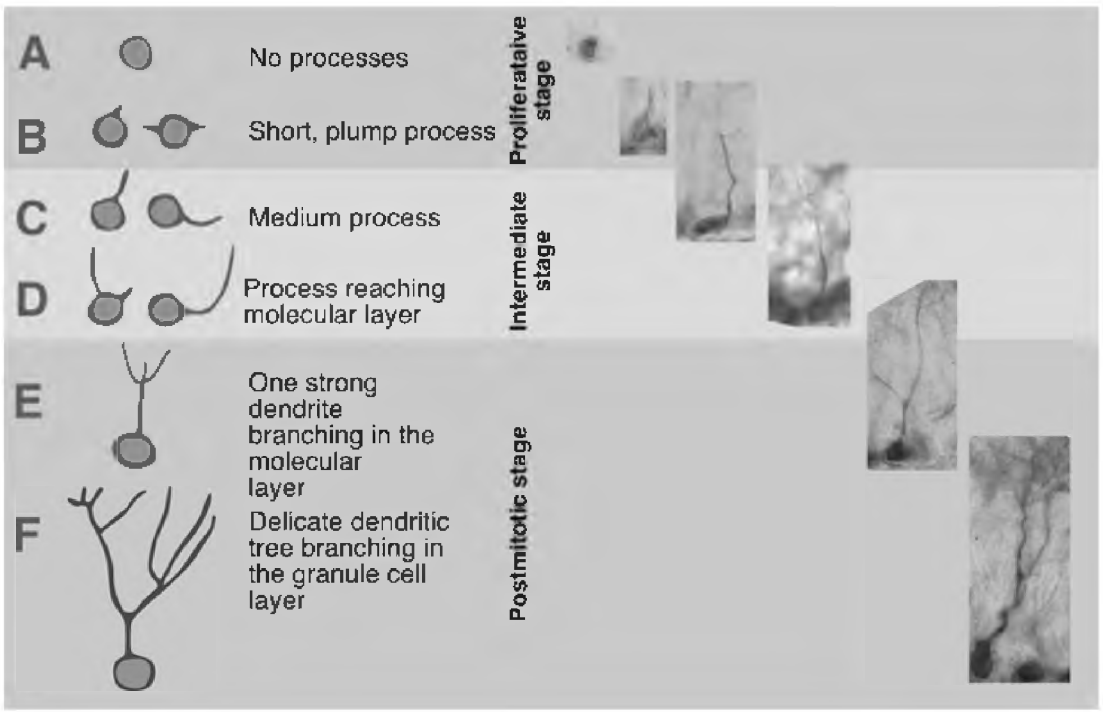


Figure 2.1: Categorization of dendritic morphology in neurons. Plumpe et al., 2006.

This is a schema of six categories of dendritic morphology describing different types of immunopositive cells seen DCX immunopositive assay. These categories A through F reflect a continuum. The right half shows light microscopic images of DCX expressing cells in the SGZ to exemplify the categories of maturation.

2.5.2 Quantitative analysis

For single labelling immunohistochemistry, quantitative analysis for Ki67, DCX, Synaptophysin and Synaptobrevin will be reported as analysed. Positive cells between groups were analysed to determine proliferation rate and neuronal survival from cell count. The total cell numbers of Ki67 and relative cell count of the DCX immunopositive neurones within the dentate gyrus of the hippocampus was analysed using the cell counter pluggin of image J software application (open source https://imagej.net). The mean values obtained, were subjected to statistical anlysis. DCX expression in each rat brain was quantified in a total of 4 serial sections which were 250 µm apart, corresponding to sections n08a – n08d of Kartens rat brain atlas. The mean value per group (standard, exercise and complex enriched environment) were analysed and compared for determination of environmental influence on proliferation rate and neuron count in the Long-Evans rat.

Double labelling immunostained cells using the Confocal based stereology (confocal co-localisation) for qualitative description of DCX positive cells was done. Qualitative and quantitative analysis of synapse and spines (types versus numbers of dendritic spine) and integrative pattern (synapse versus spine) was attempted but results were not obtained from the immunofluorescent assay of synaptobrevin and synaptophysin as the monoclonal antibodies used cross-reacted. Therefore, analysis between groups (standard, running wheel and complex enriched environment) was not possible for the determination of spine types and determination of the integrative patterns as the crossreaction distorted the slides with no image yield for analysis.

2.5.2.1 Proliferating cell count

Proliferating cells identified as Ki-67 immunopositive cells were counted in all sections on the Axiovision Light microscope using 63 X oil-immersion lens and multiplied by the section

sampling fraction to obtain the estimated total proliferating cell number in the neurogenic site of interest, the dentate gyrus.

2.5.2.2 Pyknotic (Apoptotic) cell count

Apoptotic cells were counted on the Geimsa stained sections, at a section thickness of 50µm using an estimate of the pyknotic cells. Total Cell numbers (*N*) were estimated by multiplying the number of cells counted with the inverse of the sampling fraction, i.e. *N*= sum of the cells counted × (1/thickness sampling fraction) × (1/area sampling fraction) × (1/section sampling fraction). All cell number estimates were obtained from a known fraction of the tissue and are therefore unbiased.

Generally, a substance that could dye both living and dead cells is combined with the basic principle of identifying viability of the cell from its membrane morphology to determine if plasma cellular membrane is intact or not. Thus, cells with intact cellular membranes (live cells) are differentiated from cells with defective/ absent membranes (dead cells) microscopically after staining. Apoptotic cells can be seen at different stages of the apoptotic process having spherical appearance, pyknotic appearance, absent or faded cytoplasmic membrane or a fragmented nucleolus with or without nucleoli. During both mitosis and the earlier stages of apoptosis, the cells appear spherical and smaller, but are not spread on substratum to which they are attached (Kerr et al., 1972). Blebs may be seen on apoptotic cells with careful examination and if the cells are still spread on the substratum, vacuoles develop in the cytoplasm and may also be seen (Ulakaya et al., 2011). In some cases, blisters which run over from the cytoplasm may also be observed, and these may get detached from the cell so that the interior is seen as an empty spherical structure. The detached blisters are "the ghost cells" as defined in literature (Ulakaya et al., 2011). Although membranes of the cells that proceed to apoptosis are intact at the beginning,

secondary necrosis develops during advanced stages and the membrane integrity becomes impaired. As the integrity of the membrane is impaired following secondary necrosis, the cells get stained with non-vital dyes and can be morphologically differentiated (Jain et al., 2009).

2.5.2.3 Volume of the dentate gyrus of the hippocampus

The volume of the dentate gyrus in each brain were measured using VOLUMEST application on the Image J software, on photo images taken with Axiovision light miscroscope equipped with a camera. Nuclear analysis was done on sections by counting the intact nucleus and pyknotic nucleus in each group and the results were compared across groups to estimate the nucleus ratio.

2.5.2.4 Statistical Analysis

Graph pad prism 5 statistic software and Bonferroni's multiple comparison tests were used for the statistical analysis between groups (standard, exercise and complex enriched environment). Relevant tests were then applied to generate confidence interval, relativity histogram (Shapiro-Wilks) with ANOVA for conclusive discussion on generated data. All tests were non-parametric, and $P \leq 0.05$ was considered significant.

CHAPTER THREE: RESULTS

3.0 QUALITATIVE ANALYSIS

3.1 Morphological Observation

The gross morphological findings from the Long-Evans rats exposed to the standard control, exercise and complex enriched environments are presented as follows.

3.1.1 Body weight

The body weights varied amongst the experimental groups as the standard control group had body weights ranging from 425 g to 501 g and were observed to have a mean body weight of 467 g ± 29.45, the exercise group had body weights ranging from 380 g to 495 g and were observed to have a mean body weigh of 461 g ± 42.86 and the complex enriched group had body weights ranging from 415 g to 491.3g and observed to have a mean body weight of 455 g ± 18.17. Hence the standard control group had higher mean body weight as compared to the exercise and the enriched group which were observed to have the least mean body weight. An estimate of 20 g to 40 g was observed to have been added to the body weight of each rodent every week during the exposure. However, the statistical analysis for body weights from this research showed no significant different across the experimental groups (p=0.841). See Figure 3.1.1 and Table 3.1 below.

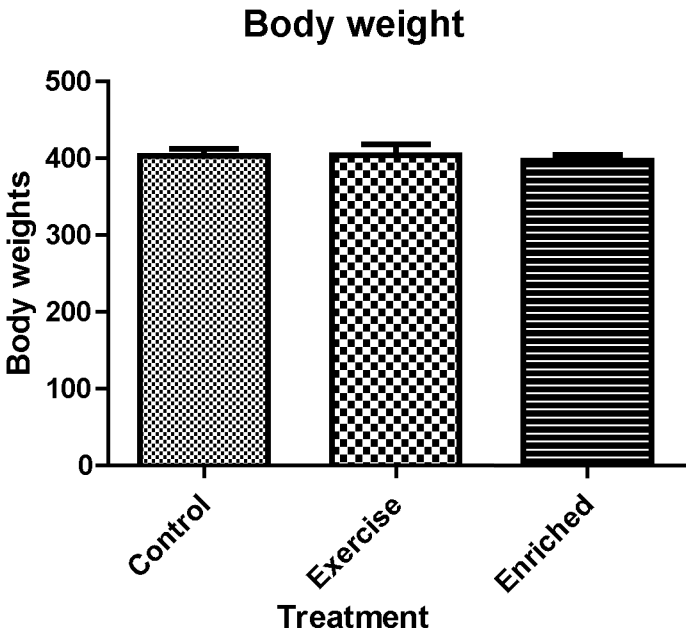


Figure 3.1.1: Bar chart of the body weight of the Long-Evans rat in the three groups

3.1.2 Brain weigth

There was variation in the brain weight amongst experimental groups. The control group had a mean brain weight of 2.033 g ± 0.052 with a range of 2.0 g in 5 animals and 2.1 g in one animal, the exercise group had a mean brain weight of 2.083 g ± 0.041 with a range of 2.0 g in 2 animals and 2.1 g in 4 animals, while the complex enriched group had a mean brain weight of 2.183 g ± 0.041 with a range of 2.1 g in 2 animals and 2.2 g in 4 animals.

The statistical analysis of the brain weights of experimental groups were found to vary significantly among groups. The enriched group had a higher brain weight when compared to the standard control and the exercise groups ($p=0.0003$). On the post-hoc analysis using Bonferroni pair wise comparison, the brain weight was found to be significantly higher in the enriched group as compared to the exercise group and also the enriched as compared to the standard control group ($p< 0.05$). There was however no significant difference observed between the exercise and the standard control groups respectively ($p> 0.05$). See table 3.1 (pg 68) and figure 3.1.2.

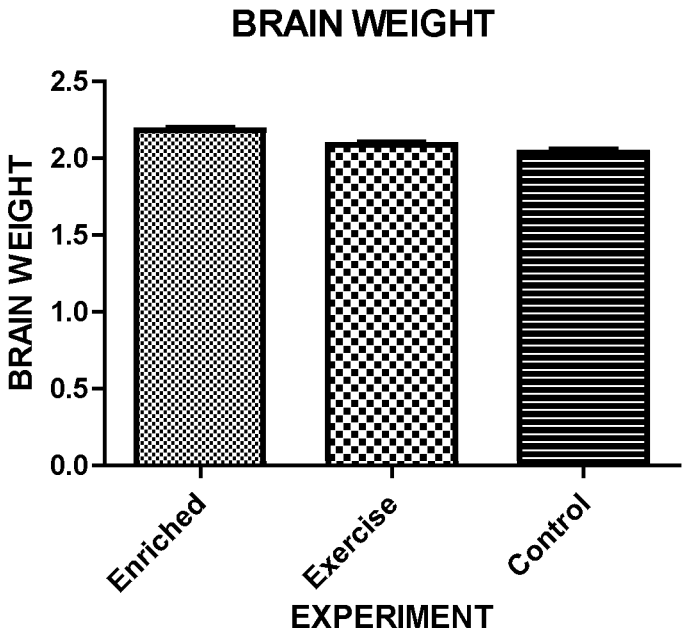


Figure 3.1.2: Bar chart of brain weight in the Long-Evans rat in the three groups.

3.1.3 Brain/Body index weight

The brain/body weight index among the experimental groups was observed to be 0.004 in the standard control, 0.005 in the exercise and complex enriched groups respectively. The statistical analysis showed no significant difference in the brain/body weight index across the experimental groups (p= 0.1661). See Figure 3.1.3 and table 3.1. The rats in the standard control group had a larger body weights and a smaller brain weight as compared to the exercise and complex enriched group which had smaller body weight and a larger brain weight.

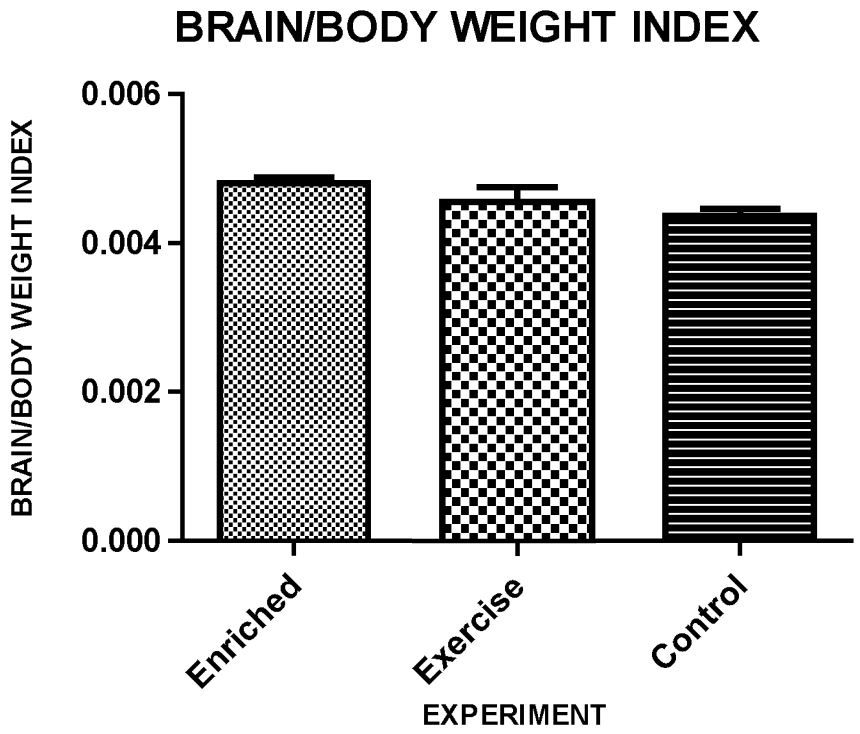


Figure 3.1.3: Bar chart of the brain/ body weight ratio in the Long-Evans rat in the three groups.

Table 3.1: Body weight and Brain weight.

	Treatments		
	Control	Exercise	Enriched
Body weight	467.5 ± 29.45	461.7 ± 42.86	455.0 ± 18.17
Brain weight	2.033 ± 0.052*	2.083 ± 0.041*	2.183 ± 0.041*
Body/Brain weight index	0.004 ± 0.000	0.005 ± 0.000	0.005 ± 0.000

Significant difference at $p<0.005$

3.2 Histology of the dentate gyrus of the hippocampus

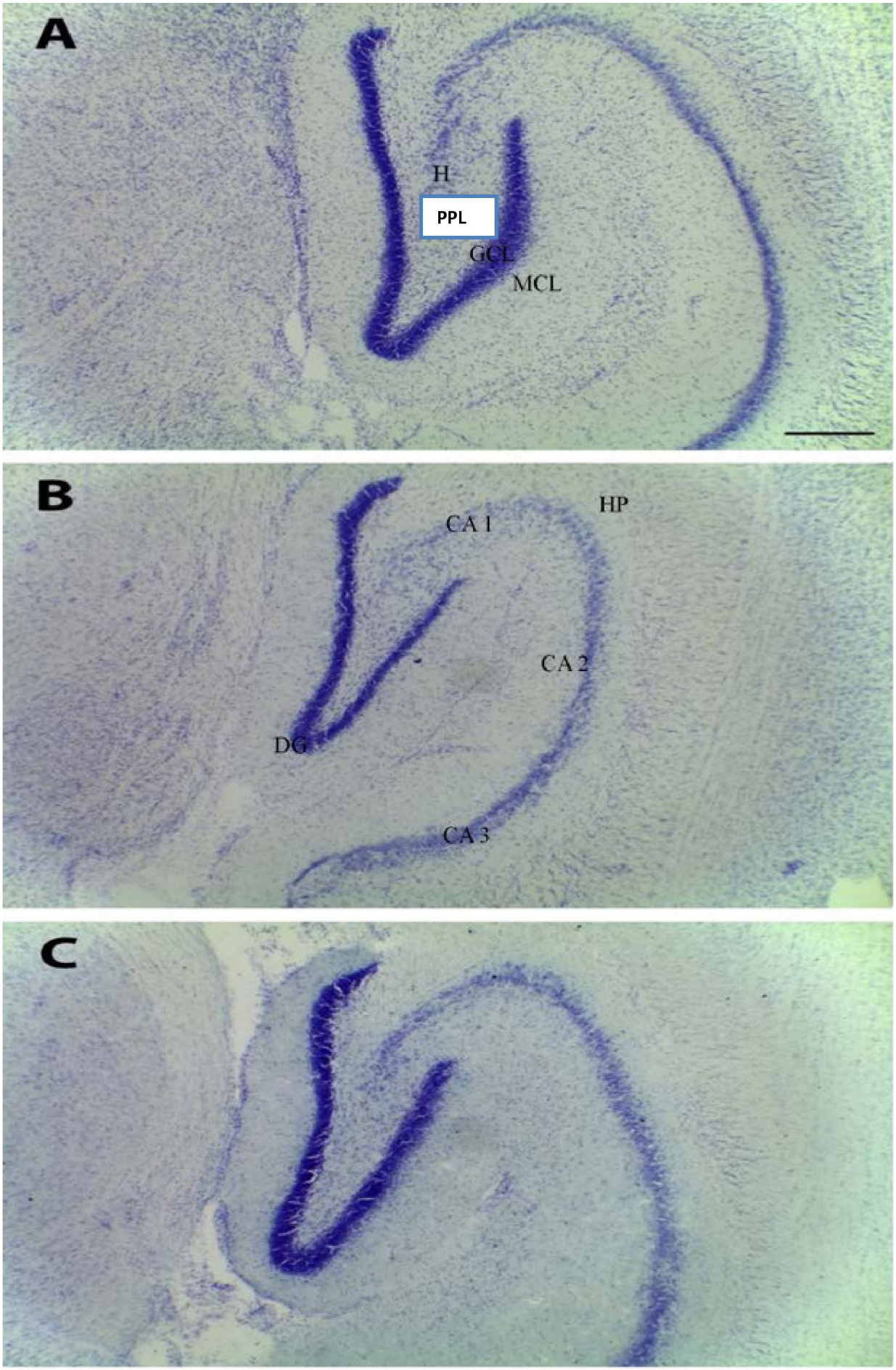
The Cresyl violet staining for nissl shows a typical rodent dentate gyrus having a dorsal and ventral limb, depicting the molecular, the granule cell and plexiform (polymorphic) layers of the dentate gyrus. There was a difference observed in the density and the compact nature of the cells on the dorsal and ventral limbs of the dentate gyrus of the hippocampus. The complex enriched group presented a more densely packed structure on the dorsal and ventral limb of the dentate gyrus of the hippocampus (see figure 3.2 A below) when compared to the exercise group which was observed to have a more densely packed ventral limb as compared to the dorsal limb of the same brain section (see figure 3.2 B below). The standard control group was observed to have a less dense dorsal and ventral limb of the dentate gyrus (see figure 3.2 C below). The hippocampus proper was observed to clearly show the typical CA regions; CA1, CA2 and CA3 regions of the hippocampus (see figure 3.2 A, B, C).

Figure 3.2: A, B, C represents Cresyl violet staining for Nissl depicting the cytoarchitecture of hippocampus of the Long-Evans rat.

Figure A (complex enriched), B (exercise), and C (standard control) show a typical rodent dentate gyrus having dorsal and ventral limbs with a plexiform layer, a granule cell layer and a molecular cell layer.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, PPL – polymorphic plexiform layer, HP - hippocampus proper, CA1, CA2 and CA3 - CA regions of the hippocampus proper

Magnification: X 50


A
H
PPL
GCL
MCL
B
HP
CA 1
CA 2
DG
CA 3
C

3.3 **Immunohistochemistry**

Immunohistochemistry results for the proliferating cells, the immature neurones, the dendrites and synapses in the Long-Evans rat employed the Ki-67, Doublecortin, Synaptophysin and Synaptobrevin markers respectively employed in the experimental groups are presented here. Immunopositive cells for each marker were observed in the dentate gyrus of the hippocampus of the experimental groups in the Long-Evans rat.

The Ki-67 positive cells were seen to be in clusters with some single cells seen infrequently within the subgranular zone of the dentate gyrus of the hippocampus. The standard control group had the least total of Ki-67positive cells counted, with an oval to spherical cell bodies. A few other pyramidal shaped cells within the subgranular zone was also noted. The exercise group had Ki-67 positive cells similar in shape and appearance to the above description, but with more dense cell clusters as compared to the standard control group. The complex enriched had Ki-67 positive cells similar also as described above, with a denser packing of the cell clusters when compared to both the standard control and exercise groups. Comparing these findings between the groups, we see a three-fold increase in Ki-67 positive cells counted between the exercise and the control, a three-fold increase between the complex enriched and the exercise and a six-fold increase between the standard control and the complex enriched group (Fig 3.3.1 A, B, C; pg 75).

The doublecortin positive cells were seen to have the cell bodies in the granule cell layer extending dendrites towards the molecular cell layer both in the single and double labelled brain sections. The standard control and exercise group had post mitotic neurones with a single dendrite arising from the soma, while the complex enriched were observed to have post mitotic neurones with two dendrites arising from a soma. The cell protrusions (primary dendrites) were seen to reach the molecular cell layer before further dividing into the secondary and tertiary dendrites in the standard control and exercise groups while the complex enriched group had the primary dendrites dividing into the secondary and tertiary before reaching the molecular cell layer (Fig

3.3.2 A, B, C; pg 79).

The synaptophysin positive cells were seen within in the hilus of the dentate gyrus of the hippocampus as round cellular droplets in clusters closely packed together in the experimental groups of the Long-Evans rat (Fig 3.3.3 A, B; pg 82).

The synaptobrevin positive cells were seen as circular droplets in seemingly linear clusters within the subgranular layer and molecular cell layer of the dentate gyrus of the hippocampus of the experimental groups in the Long-Evans rat (Fig 3.3.4 A, B; pg 85).

Figure 3.3.1: Ki-67 positive cells:

Ki-67 immunopositive cells in the dentate gyrus of the hippocampus of the Long-Evans rat were observed in the granule cell layer of the dentate gyrus in clusters of oval to round neuronal cell bodies.

Figure A (complex enriched), B (exercise) and C (standard control) show Ki-67 immunopositive cells in the dentate gyrus of the hippocampus respectively. The immonopositive cells are seen to be distributed along the granule cell layer as dark-brown staining cells in clusters and very few solitary cells.

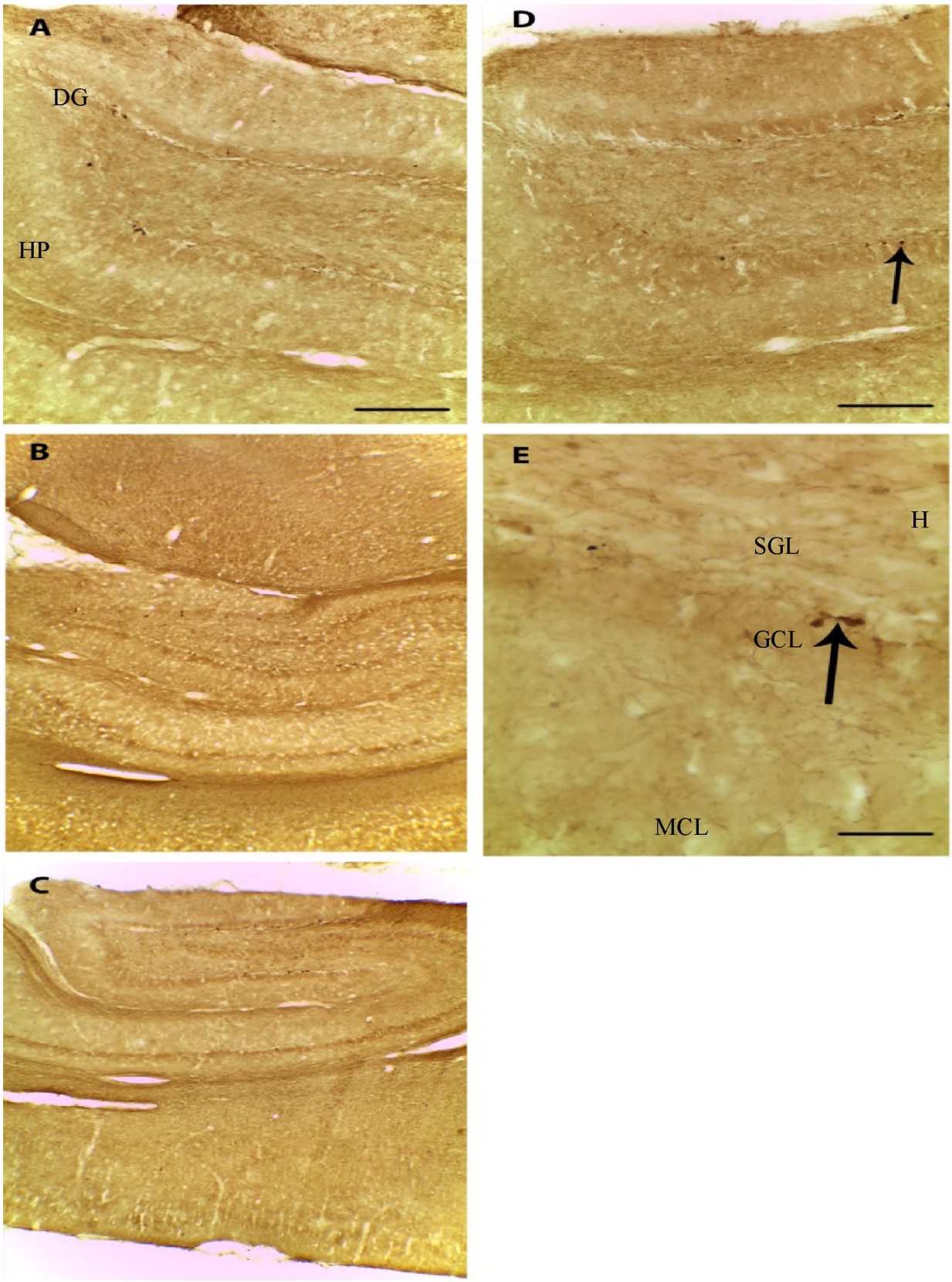
Figure D (magnification x 400) and E (magnification x 1000) from the complex enriched group to show the staining characteristics of the Ki-67 immunopositive cells.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A, B and C X 50, D X 400, and E X 1000.

Ki-67 Immunohistochemistry

Arrow; shows Ki-67 immunopositive cell in the dentate gyrus of the Long-Evans rat.


A
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B
E
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SGL
GCL
MCL
C

3.3.2 **Doublecortin (DCX) positive cells**

DCX positive cells were observed in the dentate gyrus of the hippocampus as immature neurones along with their processes (figure 3.3.2 A, B, C, D, E and F).

Single labelling immunohistochemistry using DAB showed post mitotic neurones with their cell bodies lying along the granular cell layer and the neuronal processes directed towards the molecular cell layer of the dentate gyrus of the hippocampus. DCX immunofluorescence showed DCX immunopositive neurones (green) in the dentate gyrus of the hippocampus. Control and exercise group had post mitotic neurones with more cells distributed on the ventral limb of the dentate gyrus in the exercise group as compared to the dorsal limb and having more cells in total as compared to the standard control.

Enriched versus control: Post mitotic neurones with single dendrite that extend to the MCL before further dividing into secondary and tertiary dendrites were seen in the standard control group while post mitotic neurones with two primary dendrites which began dividing into tertiary dendrites before reaching the molecular cell layer were seen in the enriched group. These neurones were more compact and evenly distributed on both ventral and dorsal limbs of the dentate gyrus in the enriched group as compared to the control as well.

Exercise versus enriched: Sections showed post mitotic neurons with a single dendrite that extends to the MCL before dividing into sec ondary and tertiary dendrites in the execise group. In addition, the neurones were more distributed over the ventral limb of the dentate gyrus as compared to the dorsal limb of the dentate gyrus in the same animal while post mitotic neurones were seen evenly distributed on both limbs of the dentate gyrus in the enriched group as described here (Fig 3.3.2 A, B, C; pg 79).

Neurone structure: The enriched group showed post-mitotic neurones that have a soma with more than one primary dendrite that began dividing into the secondary and tertiary dendrites before reaching the molecular cell layer of the dentate gyrus, while the exercise and standard group

neurones had soma with a single primary dendrite, which extends to the molecular cell layer of the dentate gyrus before further dividing into the secondary and tertiary dendrites. The enriched, exercise and standard control groups were observed to vary, in that the cell packing in the dentate gyrus, varied among the groups. The exercise group, had fewer cells on one limb (dorsal limb), in contrast to the standard control group which had sparse distribution of the immature neurons over both limbs (dorsal and ventral), while the complex enriched group had cells densely packed on both limbs (dorsal and ventral). A less complex neurone structure was also, observed in both the exercise and standard control groups while the enriched group showed a complex neurone structure with multiple branching pattern in the dendrites.

Figure 3.3.2 DCX Immunopositive cells: DCX Immunopositive cells in the dentate gyrus of the hippocampus in the Long-Evans rat.

Figure A (complex enriched), B (exercise), C (standard control) show DCX immunopositive cells respectively in the dentate gyrus of the hippocampus. Cell bodies are seen in the granule cell layer extending dendrites towards the molecular cell layer.

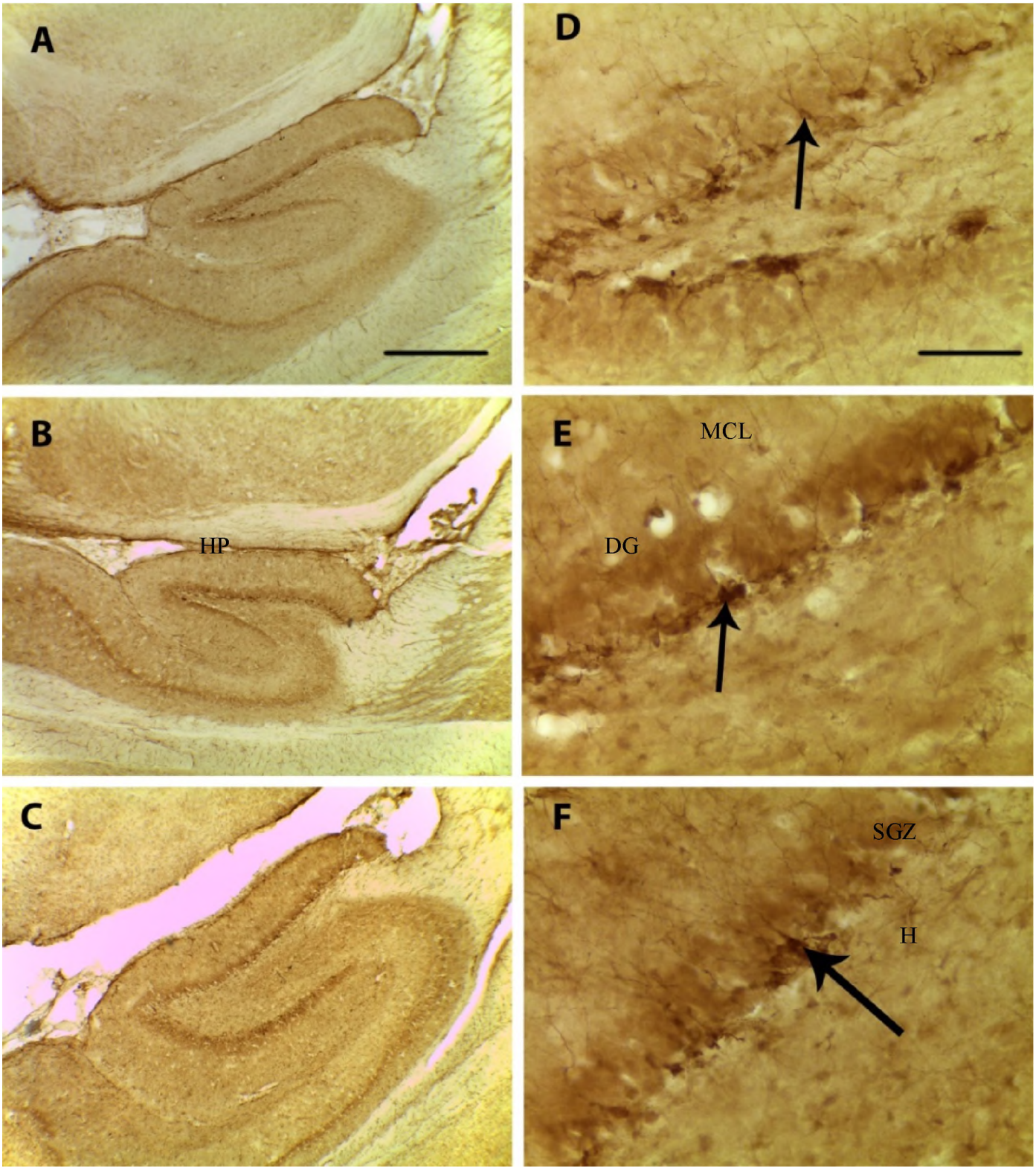
Figure D (complex enriched), E (exercise) and F (standard control) show higher magnification of the DCX positive cells and the processes.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A, B, and C X 50 and D, E and F X 1000

DCX Immunohistochemistry

Arrow; shows DCX immunopositive cells in the dentate gyrus of the Long-Evans rat.


A
D
B
HP
E
MCL
DG
C
F
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H

3.3.3 **Synaptophysin immunopositive cells**

Single labelling synaptophysin immunohistochemistry showed synaptophysin immunopositive cells in the dentate gyrus of the hippocampus of the Long-Evans rat. These cells were observed to be arranged in diffuse clusters over the subgranular cell layer of the dentate gyrus of the hippocampus. There was no variation observed in the arrangement of the positive cells between the experimental groups. These cells were not seen in low power fields in all groups (Fig 3.3.3 A, B; pg 82).

Figure 3.3.3: Synaptophysin immunohistochemistry

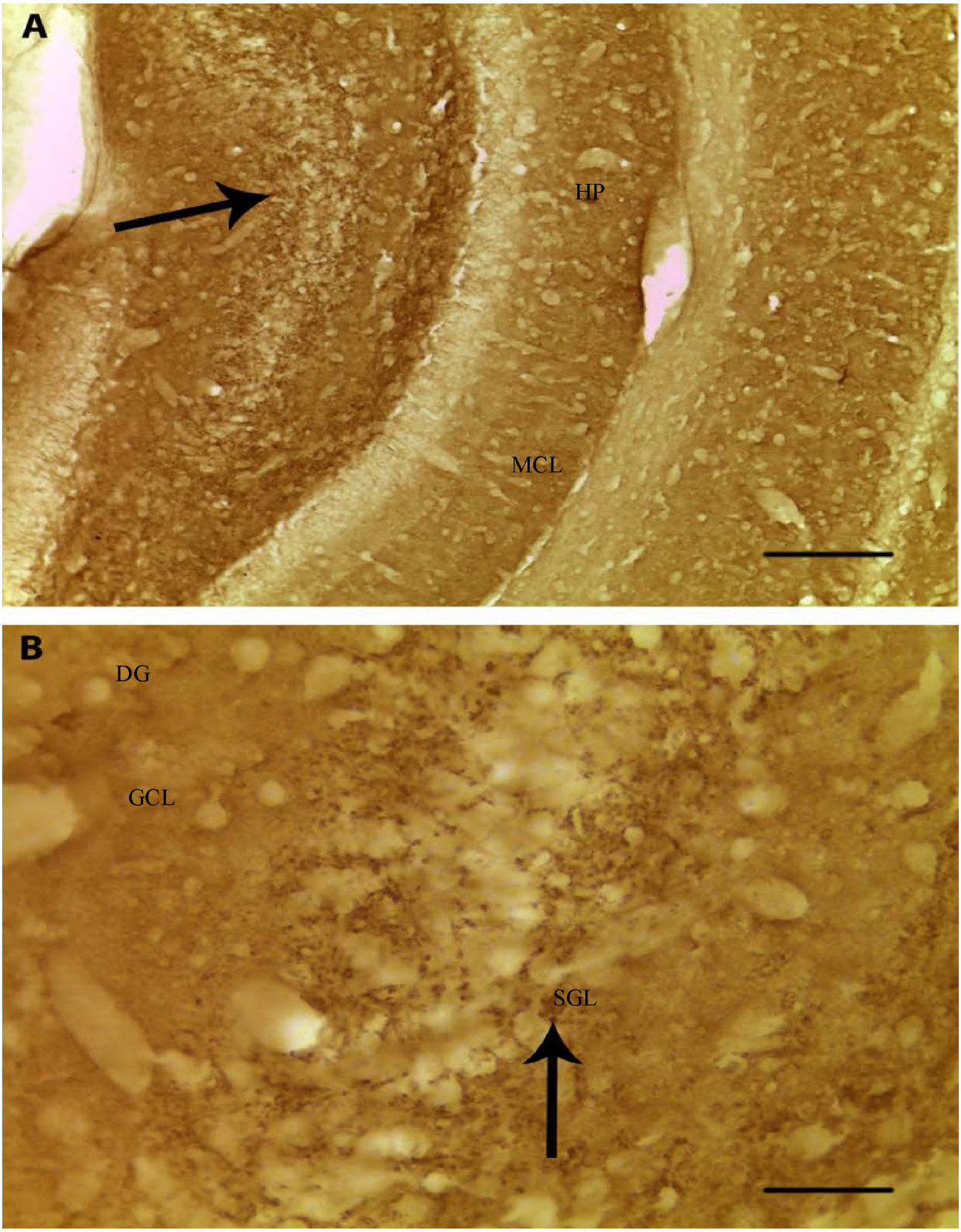
Figure A and B show synaptophysin Immunopositive cells within the subgranular zone of the dentate gyrus of the hippocampus of the Long- Evans rat. Immunopositive cells are observed to be in compact clusters.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: Slide A X 400

Slide B X 1000

Arrow; shows synaptophysin immunopositive cells in the dentate gyrus of the Long-Evans rat.


A
HP
MCL
B
DG
GCL
SGL

3.3.4 **Synaptobrevin immunopositive cell**

Single labelling synaptobrevin immunopositive cells using DAB were seen in diffuse clusters over the hilus of the dentate gyrus of the hippocampus of the Long-Evans rat. The positive cells were only seen in the high-power objective and were not observed in the low power objective in all experimental groups (Fig 3.3.4 A, B; pg 85).

Figure 3.3.4 **Synaptobrevin immunopositive cells**.

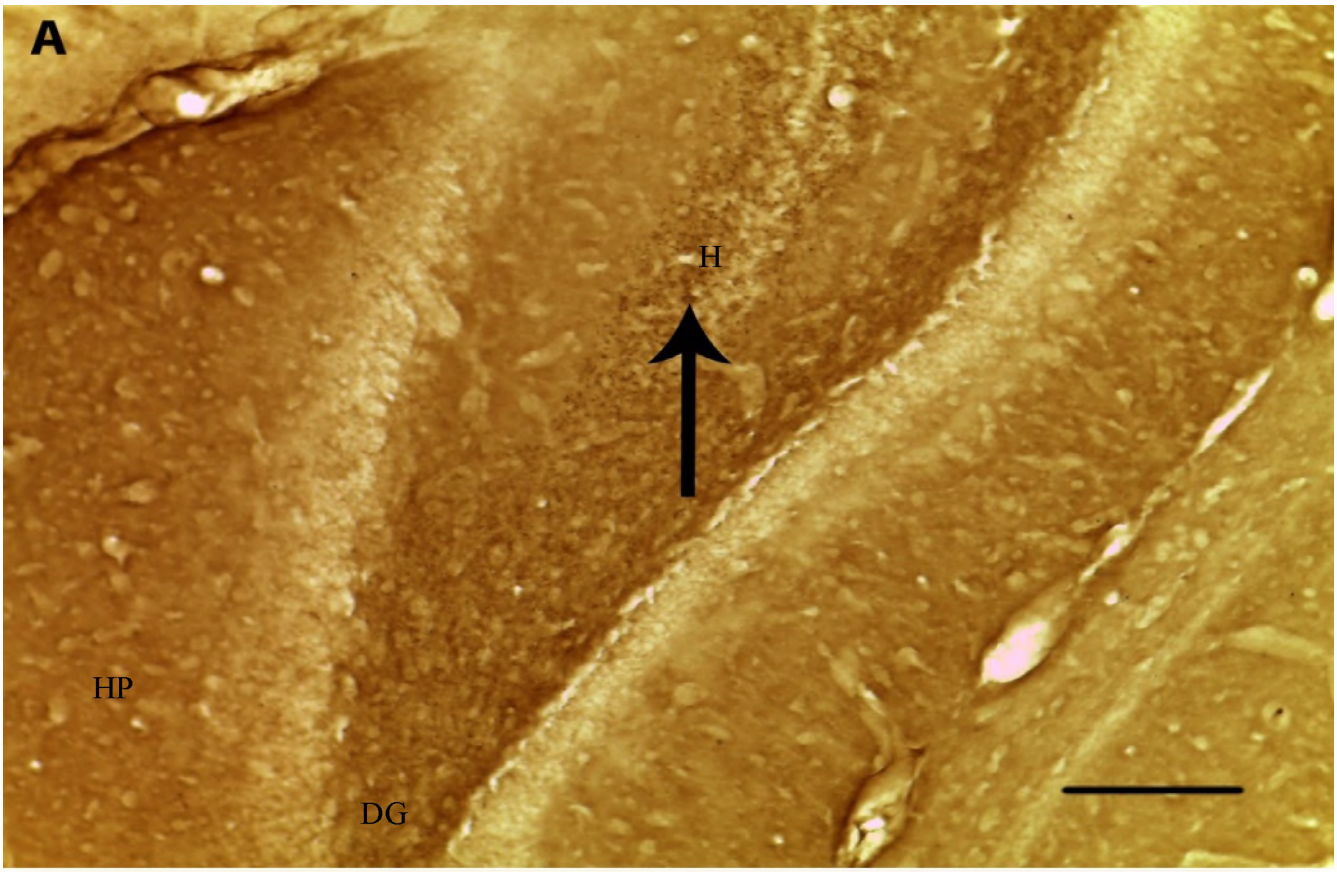
Figure A (low power) and B (high power) show synaptobrevin immunopositive cells as circular cellular droplets in diffuse clusters within the hilus of dentate gyrus of the hippocampus of the Long-Evans rat.

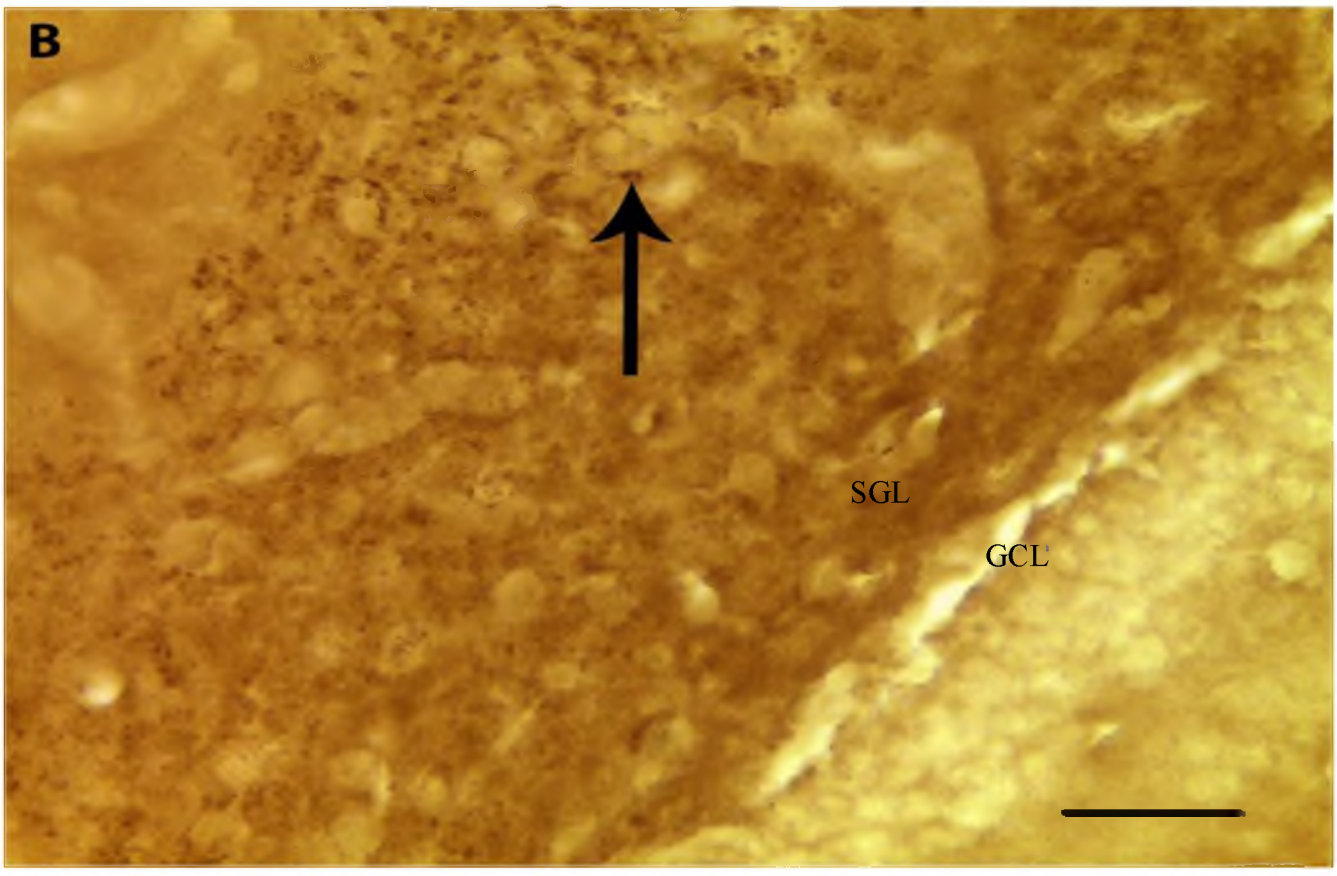
DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A X 400 and B X 1000

Synaptobrevin Immunohistochemistry.

Arrow; shows the synaptobrevin immunopositive cell in the dentate gyrus of the LongEvans rat.


A
H
HP
DG


B
SGL
GCL

3.4 **Double labelling immunofluorescence positive cells**

DCX and Synaptophysin: DCX and Synaptophysin immunofluoresent positive neurones were seen in the dentate gyrus of the hippocampus of the Long-Evans rat.

DCX and Synaptobrevin: DCX and Synaptobrevin immunofluorescent positive neurones were seen in the dentate gyrus of the Long-Evans rat.

Synaptophysin and Synaptobrevin: Syanptophysin and Synaptobrevin immunofluorescent positive neurones were not observed in any group possibly due to cross reactions between the two monoclonal antibodies.

(Fig 3.4.1., pg 88; 3.4.2., pg 90; 3.4.3., pg 92)

Figure 3.4.1: DCX, syanptophysin and Synaptobrevin Immunofluoresence positive cells in the complex enriched environment.

Figure A (DCX), B (synaptobrevin), C synaptophysin, and D (DCX neurone structure) show immunofluorescence positive neurones in the complex enriched environment. DCX positive neurones were seen along the granule cell layer projecting processes toward the molecular cell layer, Synaptobrevin positive cells within the hilus, Synaptophysin positive cells within the subgranule cell layer and DCX immunofluorescence positive neuron structure, within the dentate gyrus of the hippocampus of the Long-Evans rat.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A X 100, B, C and D X 1000.

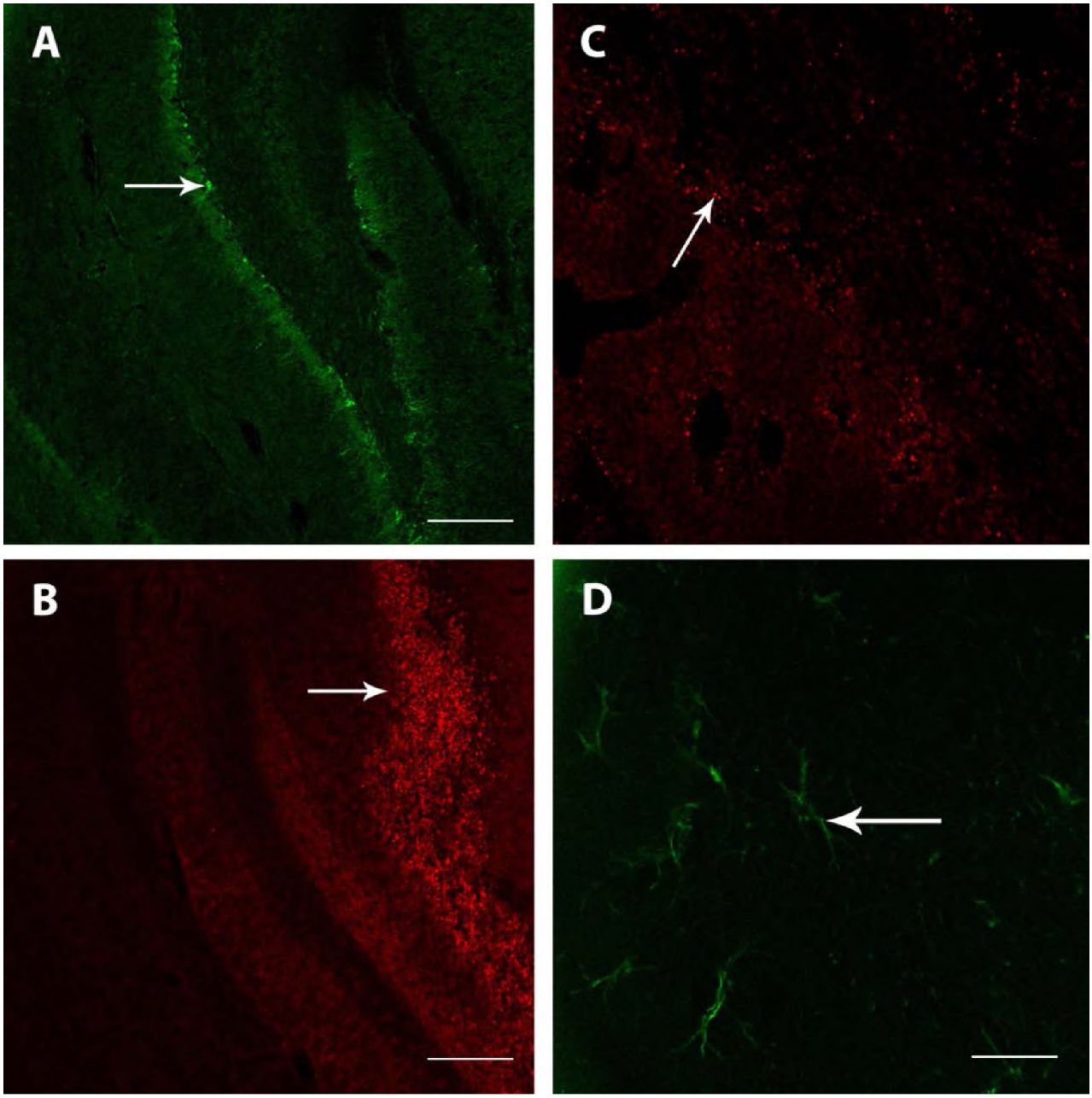

A
C
B
D

Figure 3.4.2 DCX, Synaptophysin, Synaptobrevin Immunofluoresence positive neurones in the exercise nvironment.

Figure A (DCX), B (Synaptobrevin), C (Syanptophysin), and D (DCX neurone structure) show immunofluorescence positive neurones in the exercise environment for DCX positive cells more cells on the ventral limb as compared to the dorsal limb of the dentate gyrus of the hippocampus, Snaptobrevin positive cells within the hilus, Synaptophysin positive cells within the subgranular layer and DCX immunofluorescence positive neuron structure, within the dentate gyrus of the hippocampus of the Long-Evans rat.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A X 100 and B, C, and D X 1000

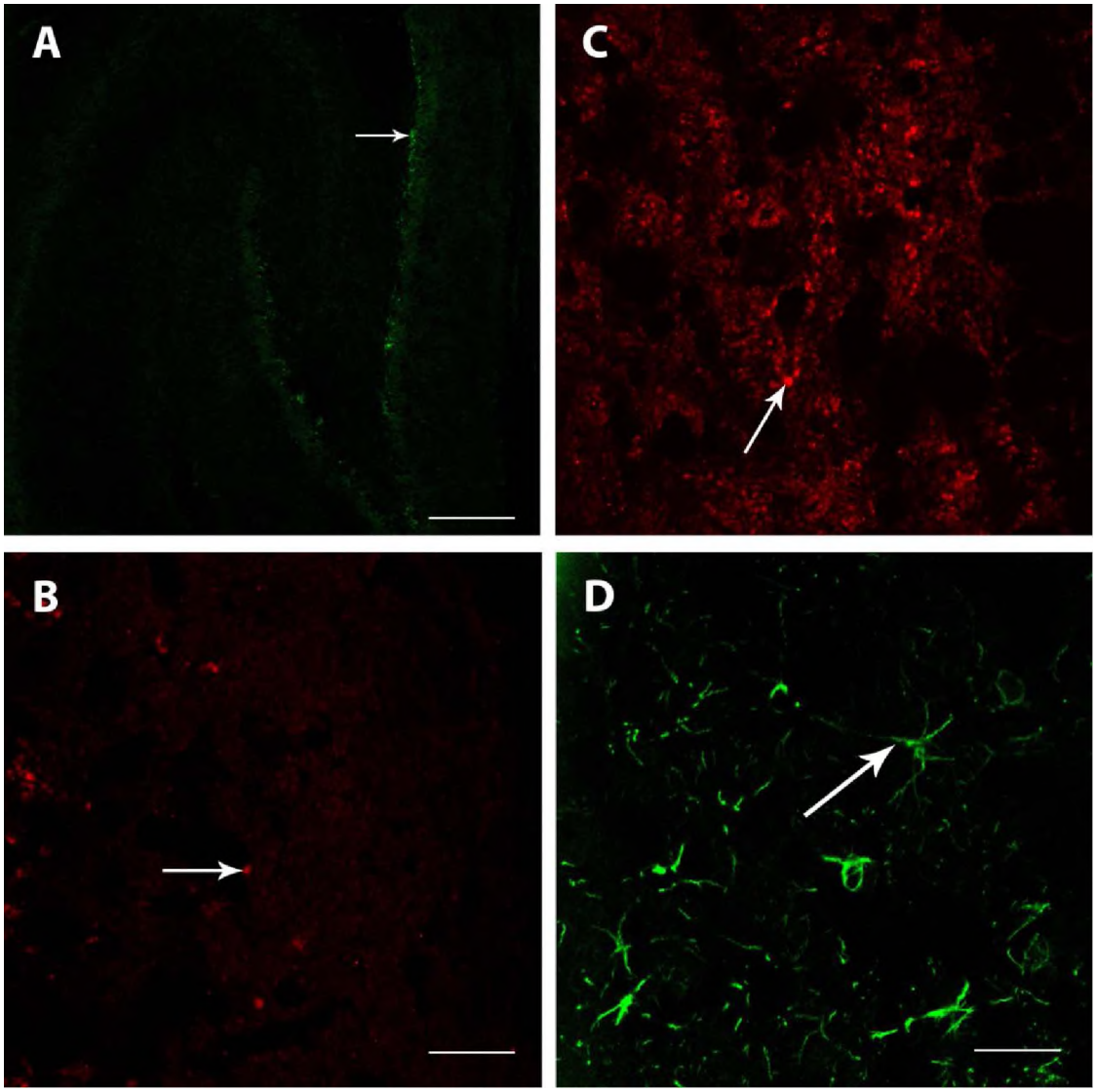

A
C
B
D

Figure 3.4.3 DCX, Synaptophysin and Synaptobrevin Immunofluoresence positive neurones in the standard control environment.

Figure A (DCX), B (Synaptobrevin), C (Synaptophysin), and D (DCX neurone structure) show immunofluorescence positive neurones in the standard control environment for DCX positive cells arranged in a scanty manner along the granule cell layer, Synaptobrevin positive cells within the hilus, Synaptophysin positive cells within the subgranular cell layer and DCX immunofluorescence positive neuron structure, within the dentate gyrus of the hippocampus of the Long-Evans rat.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A X 100, B, C and D X 1000.

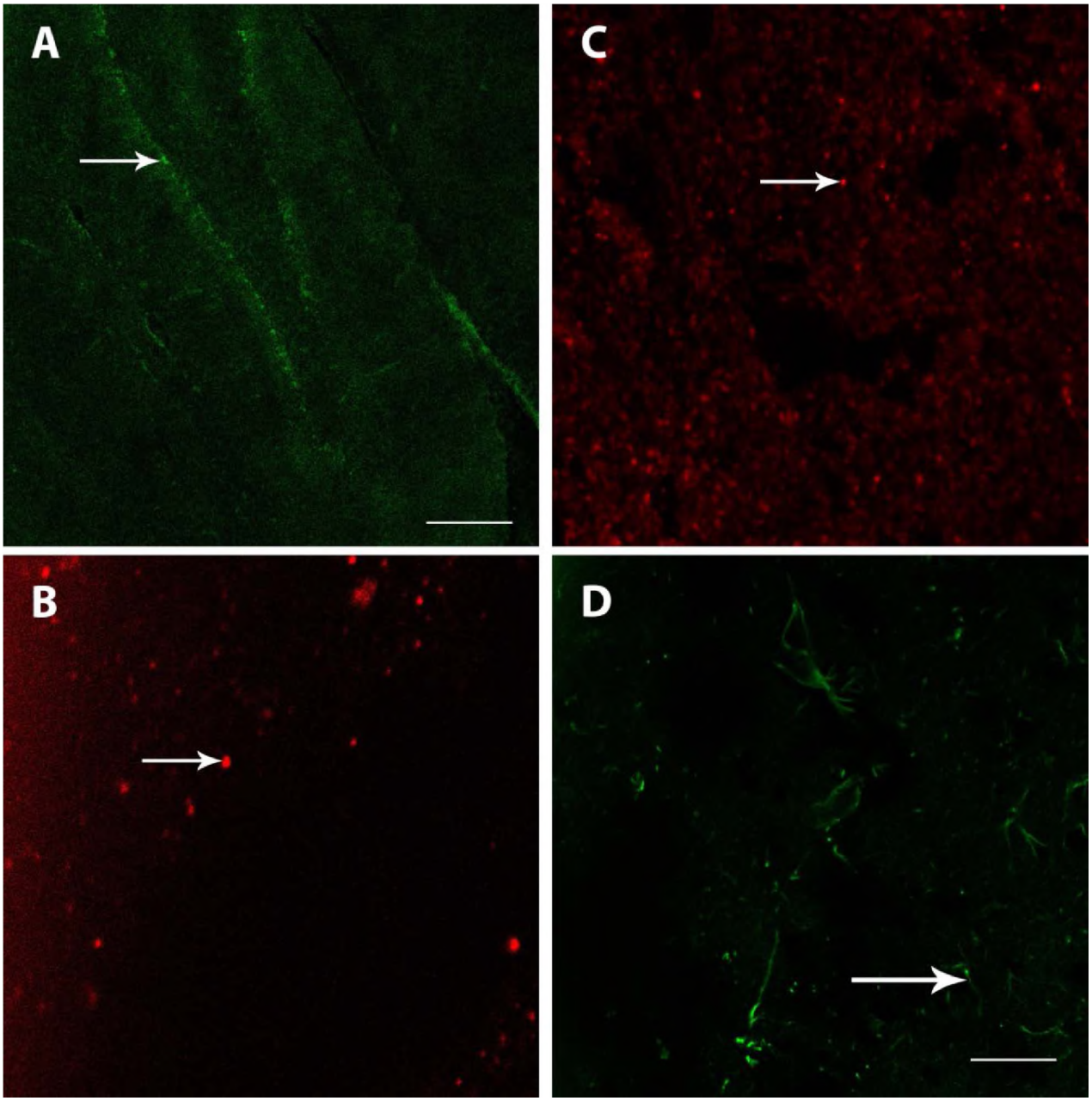

A
C
B
D

Figure 3.4.4 DCX, Syanptophysin, Synaptobrevin double labelling immunofluorescence overlay

Figure A (complex enriched), B (execise), C (standard control), and D (high power image from complex enriched) show overlay of the double labelling immunofluorescence positive neurones in in the dentate gyrus of the hippocampus of the Long-Evans rat.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A, B and C X 50, and D x 1000

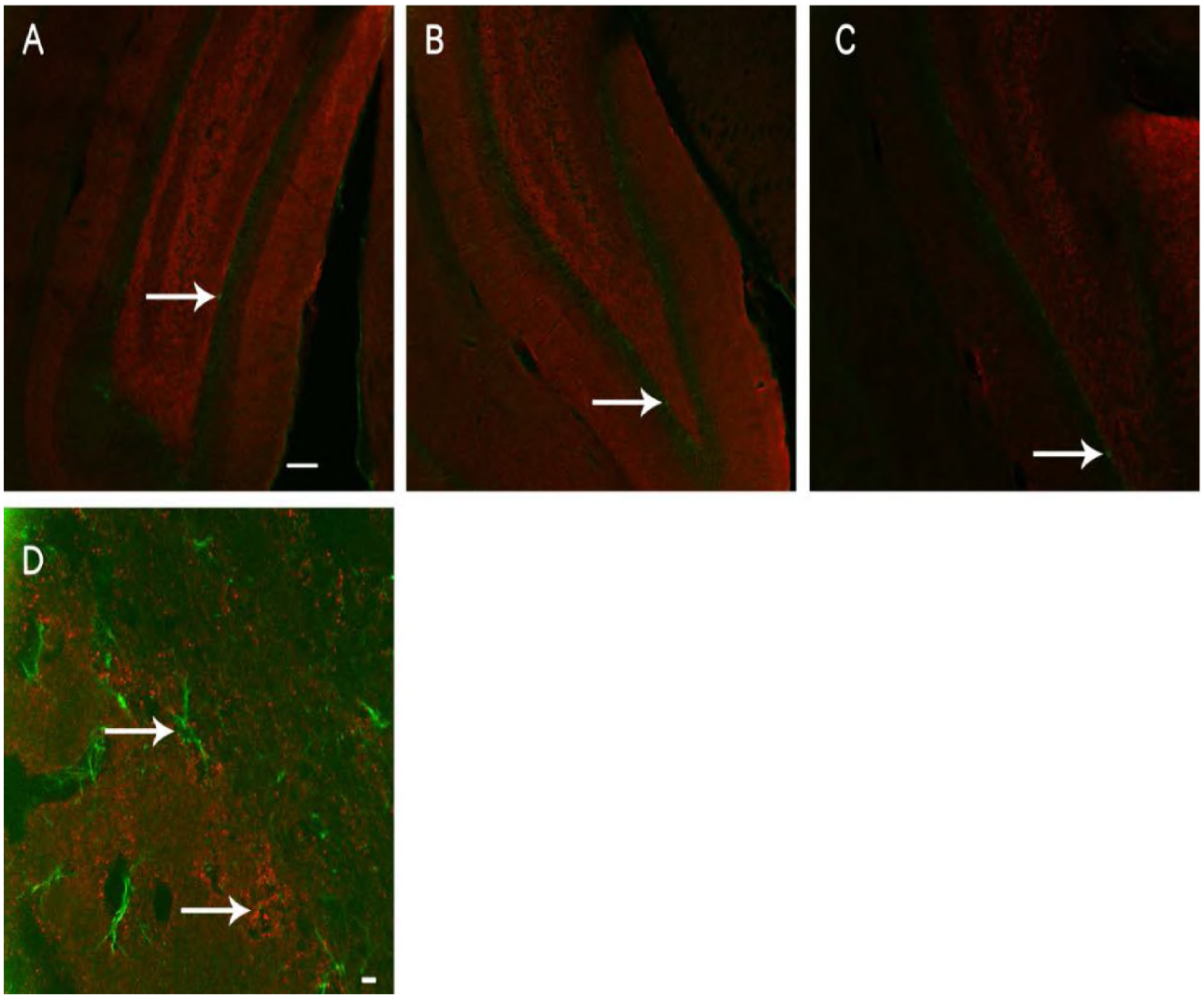

A
B
C
D

Figure 3.4.5 Negative control sections for immunohistochemistry and immunofluorescence

Figure A, B, C show negative control images for Immunohistochemistry using DAB.

Figure D (green) and E (red) show negative control images for Immunofluoresence using the green and red red fluorochromes.

In all the negative control setions, the primary antibody was omitted in the process of staining for the dentate gyrus of the hippocampus of the Long-Evans rat.

Figure 3.4.5 Ngative controls

Figure A, B, C, D and E show no positive cell as the primary antibody was omitted in all negative control sections. DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

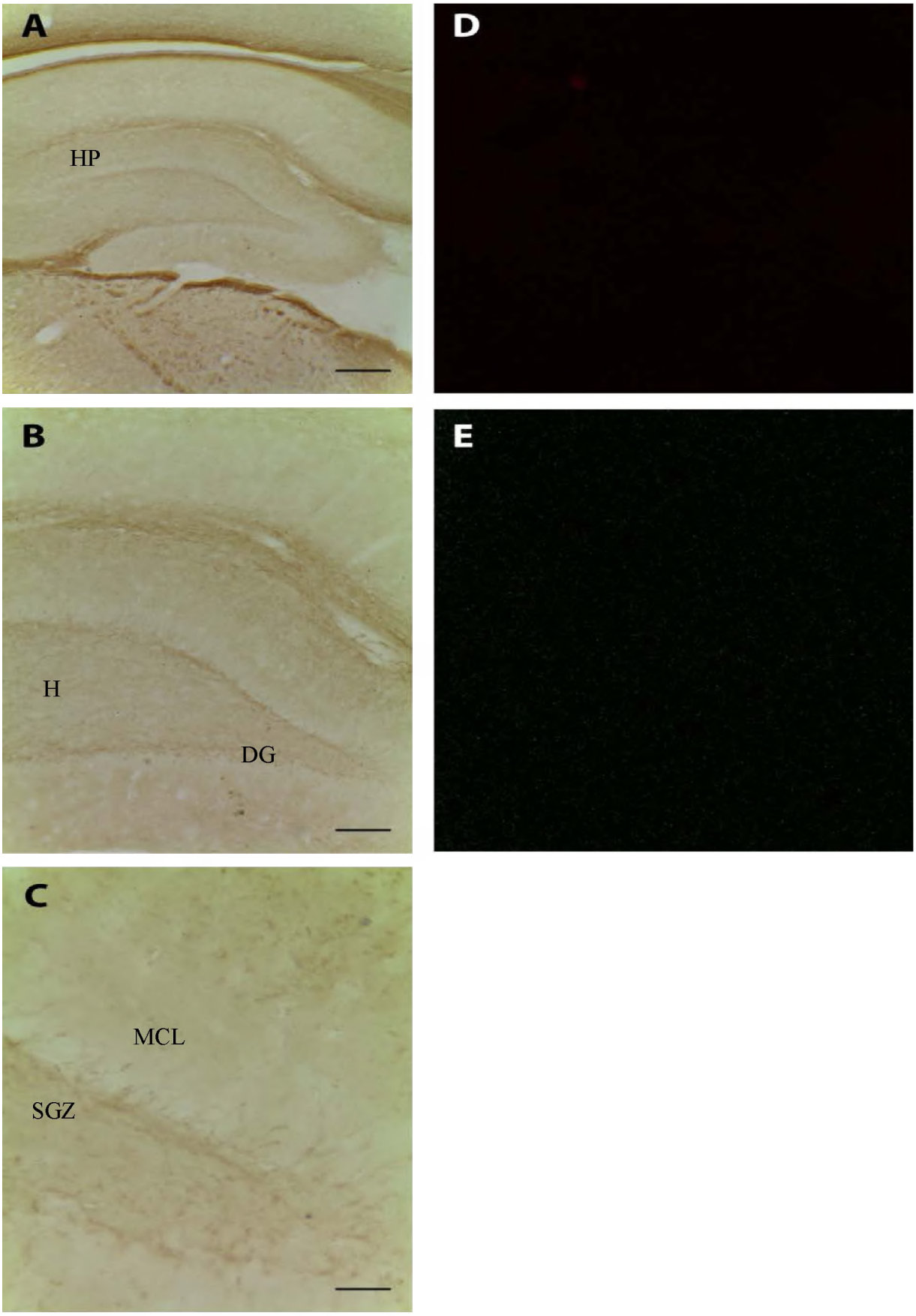
Magnification: A – DAB X 100

B – DAB X 400

C – DAB X 1000

D – Green fluorochrome X 400

E – Red fluorochrome X 400


A
HP
D
B
H
DG
E
C
MCL
SGZ

3.5 **Apoptotic cells**

Using pyknotic cell numbers on Geimsa stained brain sections of the Long- Evans rat, apoptotic cells at different phases of pyknosis in the dentate gyrus of the hippocampus of the Long-Evans rat were seen. The standard control group was observed to have a higher apoptotic cell number as compared to the other two groups (complex enriched and exercise groups).

The pyknotic cell were observed to exhibit features of pynkosis as characterised by the different stages of apoptosis as absent cytoplasm sepictind loss of the cytoplasmic membrane, faded or fading cytoplasm with or without nucleoli, fragmented nucleolus, and empty / ghost cells without nucleoli as seen in the dentate gyrus of the hippocampus of the Long-Evans rat (Fig 3.5 A, B, C; pg 100).

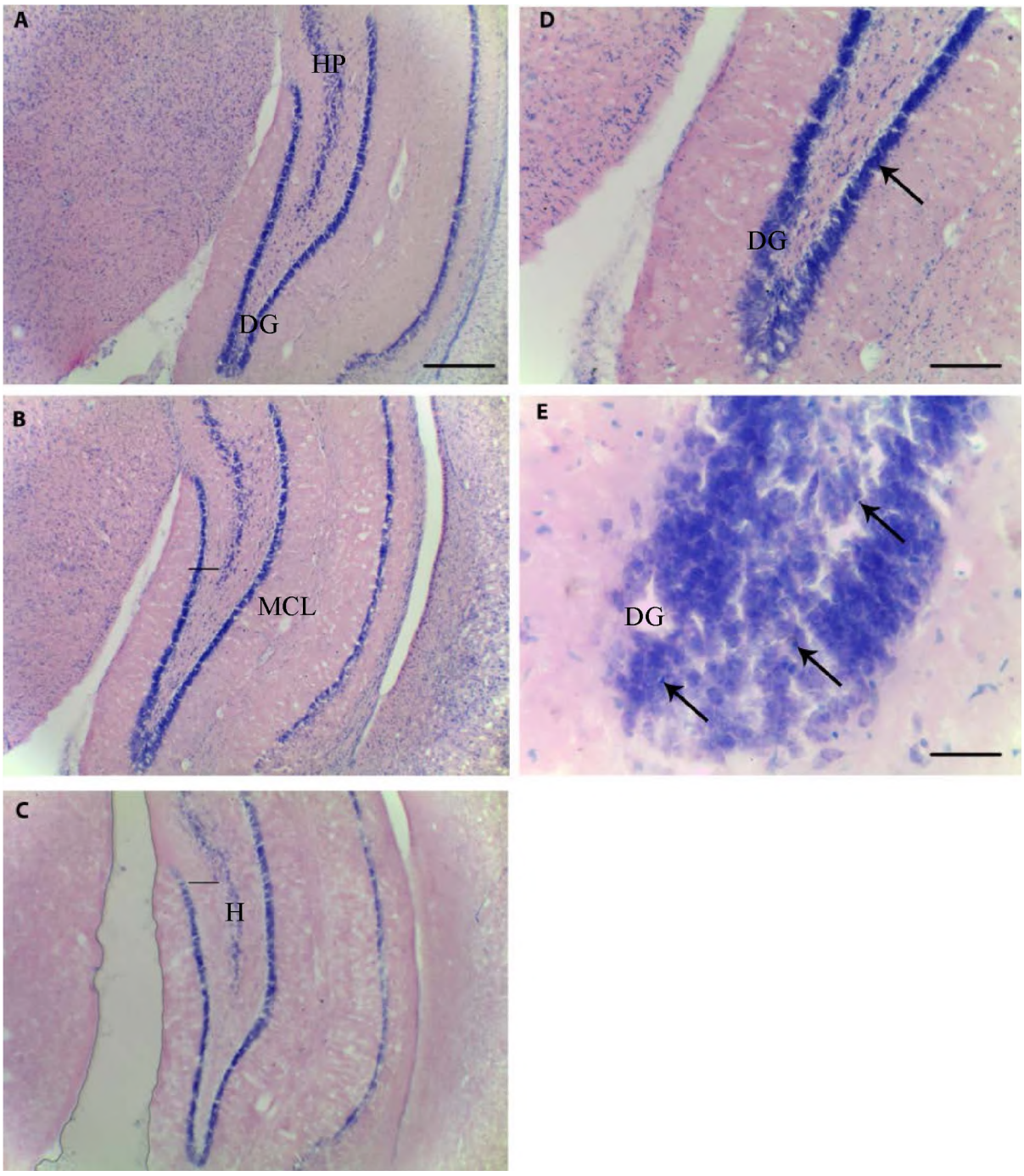
Figure 3.5 Apoptotic cells

Figure A (complex enriched), B (exercise), C (standard control), and D (high power X 40 in the complex enriched) show pyknotic cells in the dentate gyrus of the hippocampus of the Long-Evans rat stained with Geimsa.

Figure E (high power X 100 of complex enriched) show geimsa stained pyknotic cells at the different stages of pyknosis within the dentate gyrus of the hippocampus of the Long- Evans rat.

DG - dentate gyrus, MCL – molecular cell layer, GCL – granule cell layer, SGL – subgranule cell layer, HP - hippocampus proper.

Magnification: A, B and C X 100, D X 400 and E X 1000


A
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DG
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H

3.6 Quantitative and Statistical Analysis

Apoptotic / Pyknotic cell number: The mean apoptotic cell numbers counted as pyknotic cells was 5667 in the standard control environment, 5088 in the exercise environment and 2931 in the complex enriched environments respectively. See table 3.2 below

Table 3.2: Volume of the dentate gyrus, Pyknotic cell count, Ki-67 cell count and DCX positive cell numbers in the Long-Evans rat.

	Control	Exercise	Enriched
Ki-67 cell count	1160	1430	1710
DCX cell count	1295	1315	1655
Pyknotic cell count	5667 ± 328	5088 ± 318	2931 ± 159*
Volume of DG	0.009909 ± 0.002891	0.001157 ± 0.006543	0.01694 ± 0.005016

Significant difference at $p<0.005$

DG; Dentate Gyrus

3.6.1 Ki-67 Immunopositive cell count

Ki- 67 cell number: The mean cell count for Ki-67 positive cells was 1160 in the standard control environment, 1430 in the exercise environment and 1710 in the enriched environment respectively. Therefore, the standard control environment had lowest Ki-67 positive cell number, while the complex enriched environment had the highest number of Ki-67 positive cells. See figure 3.6.1 and table 3.2.

The statistical significance of the differences could however not be analysed because fewer number of animals were used (one brain per treatment group, more animals are needed for statisftical analysis) for Ki-67.

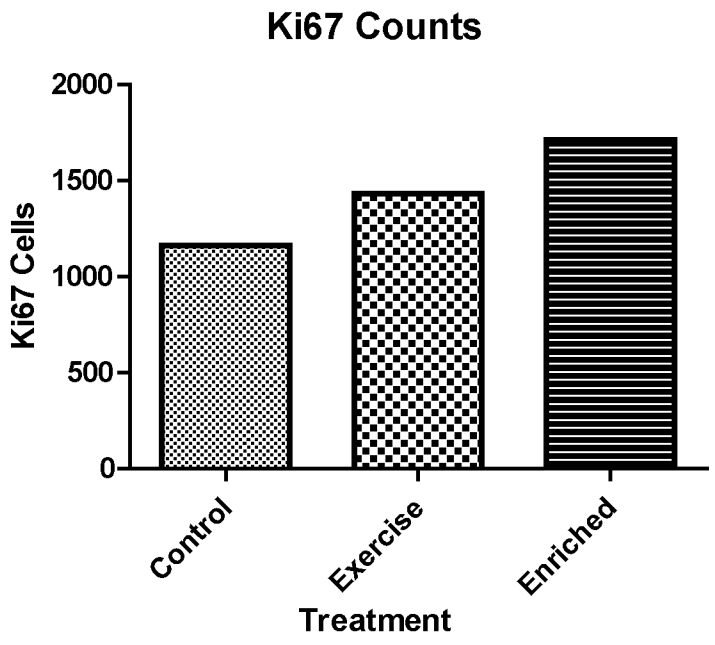


Figure 3.6.1: Bar chart for Ki-67 positive cell count in the Long-Evans rat in the three groups.

3.6.2 DCX Immunopositive cell count

DCX cell number: The mean cell count for DCX positive cells was 1295 in the standard control environment, 1315 in the exercise environment and 1655 in the complex enriched environment respectively. Therefore, the standard control environment had lowest count for DCX positive cell numbers, while the complex enriched environment had the highest number of DCX positive cells. See fig 3.6.2 and table 3.2.

The statistical significance of the differences could however not be analysed, as fewer number of animals were used (one brain per treatment group, more animals are needed for statistical analysis) for the DCX immunohistochemistry.

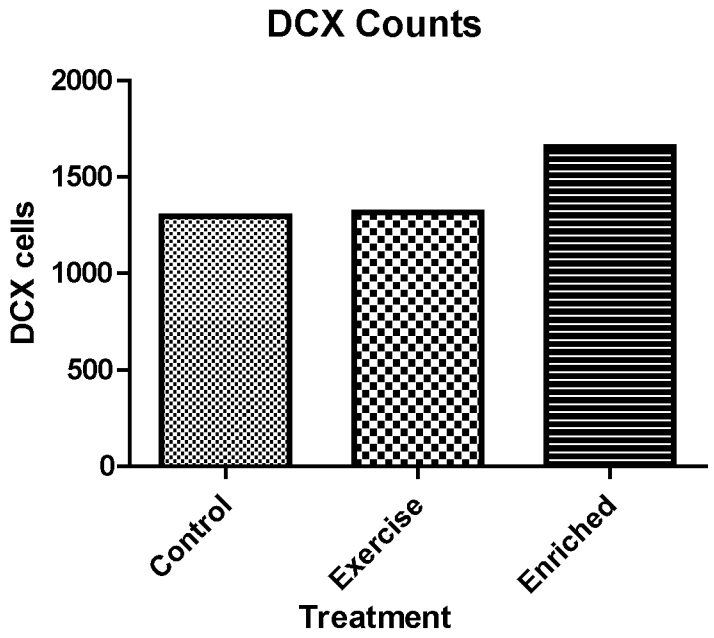
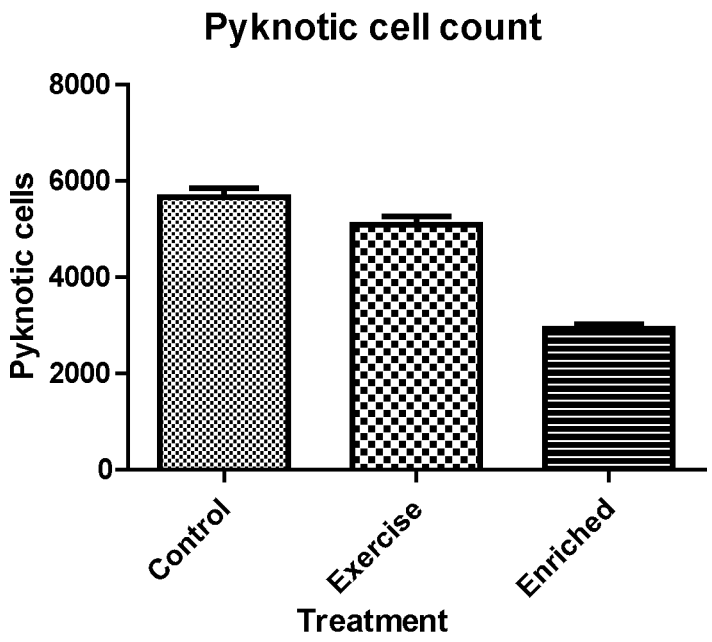


Figure 3.6.2: Bar chart for DCX positive cell count in the Long-Evans rat in the three groups

3.6.3 Apoptotic/Pyknotic cell count

The pyknotic cell number was significantly higher in the standard control environment with 5667 apoptotic cells, the exercise environment had 5088 apoptotic cells and the complex enriched environment had a total of 2937 apoptotic cells at p= 0.0010. See figure 3.6.3 and table 3.2

On post-hoc analysis using the Bonferoni pair wise analysis, the pyknotic cell number was found to be significantly higher in the control group compared to the enriched group ($p < 0.05$); the pyknotic cell number was also observed to be higher in the exercise group compared to the enriched group ($p < 0.05$). In addition, the pyknotic cell number was however not significantly different between the control and exercise groups ($p > 0.05$). See table 3.2.



($p < 0.05$) *

Figure 3.6.3: Pyknotic cell count in the Long-Evans rat in the three groups

3.6.4 Volume of the dentate gyrus in the Long-Evans rat

Volume of the dentate gyrus: The volume of the dentate gyrus analysed was 9909 x 10^6 m^3 in the standard control environment, 1157 x 10^5 m^3 in the exercise environment and 1694 x 10^5 m^3 in the complex enriched environment respectively. Therefore, volume of the dentate gyrus analysed for the dentate gyrus of hippocampus of the experimental groups in the Long-Evans rat was found to be smaller/ least in the standard control environment while it was larger in the complex enriched environment. See table 3.2

There was no significant difference observed in the volume of the dentate gyrus across the experimental groups p= 0.0968 in the dentate of the Hippocampus of the Long-Evans rat. See Fig 3.6.4 and table 3.2.

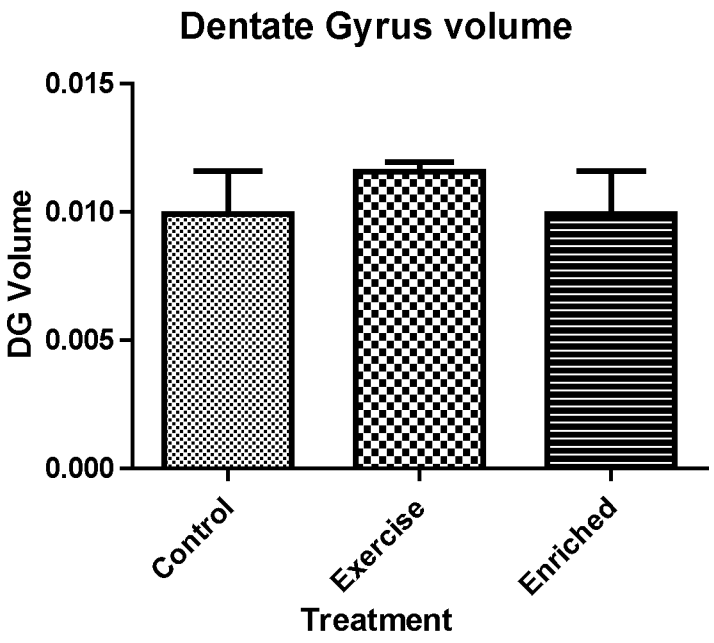


Figure 3.6.4: Volume of the dentate gyrus of the Long-Evans rat in the three groups

Table 3.3: Summary Statistics of the experimental groups.

Treatment						
Variables	No. of animals	Average No of sections	Control (Mean ± SD)	Exercise (Mean ± SD)	Enriched (Mean ± SD)	Sig. diff
Body weight	6	N/A	467.5 ± 29	461 ± 43	455 ± 18	No
Brain weight	6	N/A	2.03 ± 0.052	2.08 ± 0.041	2.18 ± 0.041*	Yes
Ki-67 cell count	1	21 x 3	1160	1430	1710	No
DCX cell count	1	21 x 3	1295	1315	1655	No
Pyknotic cell count	3	21 x 9	5667 ± 328	5088 ± 318	2931 ± 159*	Yes
Volume of DG	3	21 x 9	0.009909 ± 0.002891	0.01157 ± 0.006543	0.01694 ± 0.005016	No

* mean variables are significantly different ($P<0.05$)

SD = Standard Devaiation.

DG = Dentate Gyrus

CHAPTER FOUR: DISCUSSION

4.1 Adult neurogenesis

Granule cell precursors exist in the hilus and subgranular zones of the DG and the proliferating precursors of the granule cells are multipotent (Gould 1999). These newly generated neurones within the subgranular zone migrate a short distance as neuroblasts and become integrated as granule neurones within the granular cell layer of the dentate gyrus (Eriksson et al., 1998; Kempermann et al., 2004a). Throughout life, granule cells are continuously added to the dentate gyrus of the hippocampus (Altman and Das 1965; Eriksson et al., 1998). We found these to be true from the results of our research, as immunopositive cells were found within the hilus, subgranular zone, granular zone, and also the molecular cell layer of the dentate gyrus in the Long-Evans rat.

The body weights were higher in the standard control group compared to the exercise and complex enriched groups. This observation can be directly related to the sendentary nature of the standard control environment where the rats were engaged in minimal activity. In the activity driven environments weight gain was minimal. In general, the rodents were observed to have added between a minimum of 20 g to a maximum of 40 g per week, higher weight gain per week seen in the standard control group supporting the phenomenon that activity and exercise regulates weight, burns fat and improve basal metabolic rate. Exercising helps people reduce weight maintain weight and fights obesity (A.D.A.M inc., 2017). Weight changes (increase or decrease) are affected by the amount of energy expended versus the amount consumed, hence when expenditure is low and consumption remains high weight gain occurs from storage of the excess (Damon et al., 2014).

The rats in the complex enriched environment were noted to be more social when compared to the behaviour of the other experimental groups. In addition, the spatial distribution and cell packing of the immature cells on the limbs of the dentate gyrus are specific to the functions promoted by the enrichment paradigm. The running wheel used for the exercise group was suspended in space at

the free end of the cage, implying that rats had to learn how to oriente themselves in space as they ran as this encouraged cell growth on the dorsal arm of the dentate gyrus of the hippocampus compared to other experimental groups.

4.2 Neurone Structure

The neurone structure seen are post mitotic neurones. In the complex enriched environment group, there were post mitotic neurones of category F (Plumpe et al., 2006). These neurons had a more complex structure, with most of the soma having more than one primary dendrite (the basal dendrite) which began to divide into the secondary and tertiary dendrites (apical dendrites) before reaching the molecular cell layer of the dentate gyrus of the hippocampus. The exercise environment and standard control environment had post mitotic neurones of category E (and Plumpe et al., 2006). These neurones had a soma from which a single primary dendrite (the basal dendrit) projects, and these dendrites extended up to the molecular cell layer of the dentate gyrus of the hippocampus before further dividing into the secondary and tertiary dendrites (apical dendrites). Similar reports exist in literature on granule cells of the dentate gyrus of the hippocampus in other animals (Davis and Macrides 1981; Luskin and Price 1983; Mori et al., 1983; Mori 1987; Chen et al., 2000; Halabisky and Strowbridge., 2003; Balu et al., 2007). The implication is that, the more cells are stimulated to mature into neurones, they form stronger network with possibly faster processing speed when this is related to the functional relevance of these new cells.

In this research, a consistent observation was that substantial number of new cells are generated in the dentate gyrus following adult neurogenesis (but only a small subset) of the newly added cells survived, matured into neurons (and possibly integrated) into the existing neuronal circuitry. This is consistent with documented reports in literature (Cameron and McKay, 2001; Kempermann et al., 2003; von Bohlen Und Halbach., 2007). In addition, enrichment and exercise positively

influenced adult hippocampal neurogenesis in the dentate gyrus of the hippocampus in the Long-Evans rat by improving cell survival and maturation into neurons (as also reported in literature that activity dependent neuronal survival promotes adult hippocampal neurogenesis. Our results further suggest that adult hippocampal neurogenesis may be an adaptive process in response to environmental and/or internal challenges (Lledo et al., 2006). In literature, various factors influence distinct stages of adult hippocampal neurogenesis including cell proliferation, neuronal differentiation and their survival (Kempermann et al., 1997; Kempermann et al., 1999a; van Praag et al., 1999b) and some observations from this research finds supporting evidence in favor of enrichment and activity driven processes incorporated into the enriched and the exercise environments. Futhermore, increased environmental complexity (relative to standard housing as control) - by enhancing the opportunities for social, cognitive, sensory and motor stimulation was found to increase hippocampal cell proliferation, differentiation, survival and maturity in the Long-Evans rat.

Voluntary physical activity (using the running wheel) was also found to improve hippocampal adult neurogenesis as it increased cell proliferation, neuronal differentiation and survival in the dorsal limb (spatial limb) of the dentate gyrus of the hippocampus in the Long-Evans rat), as also reported in literature (van Praag et al., 1999a; van Praag et al., 1999b; van Praag et al.,2005; Zhao et al., 2006). van Praag et al (1999a) separately investigated components of environmental complexity found that one component (physical activity) is associated with enhanced hippocampal neurogenesis. Also, literature suggest that voluntary physical activity and complex environment increase vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF), which in turn influence hippocampal neurogenesis positively (Fabel et al., 2003; Farmer et al., 2004).

In considering the dichotomic organization of these networks into functional compartments, the ventral hippocampus subserves for memory and learning encompassing control of attention,

executive functions, processing speed and mood while the dorsal hippocampus subserves for cognitive functions of spatial processing encompassing involvement in the regulation of stress, emotion and affect. The functional dichotomy was observed in the dentate gyrus of the hippocampus of the Long-Evans rat in the neuronal arrangements on the dorsal and ventral limbs of the dentate gyrus (Fanselow and Dong 2010; Deng et al., 2010).

There was wide spread distribution of the immature neurons on both limbs (dorsal and ventral) of the dentate gyrus in the complex enriched environment when compared to the exercise environment, which had more immature neurons distributed on the dorsal limb than the ventral limb. This supports that spatial memory is on the dorsal limb theory, as the running wheel used for the exercise environment, was suspended at the free end of the cage. Therefore, required the Long-Evans rats to orientate themselves as they ran in the suspended wheel.

The neuronal projections terminate at regions where we expect to find the dendritic and axonal terminals. From this study the dendritic terminals (stained with synaptobrevin) were seen in the molecular cell layer and the hilus of the dentate gyrus of the hippocampus, while the synaptic terminals (stained with synaptophysin) were seen in the subgranular zone of the dentate gyrus in the Long-Evans rat.

Apoptosis, a process of planned cell death, is a natural event necessary for the survival of multicellular organism. Apoptotic cell count using pyknotic cells on Giemsa stained sections elicited dead cells in the dentate gyrus of the Long-Evans rat. Apoptotic cells in the dentate gyrus of the Long-Evans rat in this research showed pyknotic cells at different stages of apoptosis having spherical or irregular appearance, an absent or faded cytoplasmic membrane with or without nucleoli and fragmented nucleolus (Jain et al., 2009; Ulakaya et al., 2011). The standard control environment was found to have a higher apoptotic cell count (about a five-fold increase) when compared to the exercise group and a twenty-six-fold increase when compared to the complex enriched environment. These differences were statistically significant ($p=0.05$).

The observed increase in the apoptotic count found in the standard control environment, suggest that more of the proliferating neurones did not progressively differentiate and mature into neurones, hence the high count. In as much as they might have had similar proliferation rates with other environments investigated in this research, due to a lack of activity to stimulate the cells through enrichment, they seemingly failed to progress when compared with the exercise environment, as a single influencing factor and the complex enriched group as a multiple influencing factor, having various activities as stimulating factors.

The exercise environment indicated a five-fold decrease in apoptotic cells when compared to the standard control. A twenty-one-fold decrease in the apoptotic cell numbers was observed in the complex enriched environment when compared to the standard control. This can be related to the influence produced from the nature of the environment when compared to the multiplicity of the complex enriched environment.

The shape and volume of the dentate gyrus was also observed to vary between the dentate gyrus of the hippocampus in the Long-Evans rat. In the complex enriched environment group, the dentate gyrus of the hippocampus was observed to be a robust V-shape with the dorsal and ventral limb almost equal. The exercise environment group was seen to have a dentate gyrus of the hippocampus with a V-shape whose dorsal limb was more robust and longer than the ventral limb, while the standard control environment group was observed to have a dentate gyrus that is V-sahped and slender on both limbs (dorsal and ventral) and also almost equal in length. This implies that the nature of enrichment is invariably important as cell stimulation is function dependent. It also suggests that the limbs vary in function and response to various factors as earlier mentioned with regards to the structural and functional dichotomy of the limbs of the dentate gyrus.

Synaptophysin immunopositive cells were noted in all experimental groups as cellular droplets, in compact cell clusters over the subgranular zone and the molecular cell layer of the dentate gyrus.

A notable difference (descriptive; as the differences was not tested), in the cell packing nature as of the dentate gyrus was observed. Here, the standard control was seen to be sparsely packed, while the complex enriched was most densely packed with the synaptophysin immunopositive cells. Although a visible difference was noted, the cells were not quantified amongst the experimental groups and hence statistics to note the level significance of the observed difference could not be analysed. This was because the section thickness of 50 μm was too thick making it difficult to focus a z-layer. From this result, we can therefore imply that, environmental enrichment and exercise improves with a positive effect on the integration phase of the new born neurones of adult neurogenesis, in the dentate gyrus of the hippocampus of the Long-Evans rat.

Synaptobrevin immunopositive cells were similarly observed in all experimental groups. They were seen in cell clusters over the hilus of the dentate gyrus of the hippocampus in the Long-Evans rat. A notable difference (observational as well), was seen in the cell packing nature of the clusters. We observed, fewer cellular droplets in the clusters found in the standard control group when compared to the densely-packed cell clusters of the complex enriched environment. These were noted over the hilus of the dentate gyrus in the Long-Evans rat. Similarly, the cells were not quantified (due to difficulties posed by the section thickness), thus no statistical analysis was done to determine the level of significance of the differences observed in the analysed groups. This result thus implies that, environmental enrichment and exercise positively influences the integration phase of adult hippocampal neurogenesis in the dentate gyrus of the Long-Evans rat.

CHAPTER FIVE: CONCLUSION AND FUTURE RESEARCH

5.1 Conclusion

This study has shown evidence of adult neurogenesis in the Long-Evans rat using Ki-67, DCX, Synaptophysin, Synaptobrevin and apoptosis using Geimsa stain in the dentate gyrus of the hippocampus. The effects of environmental enrichment and exercise having well-established evidence, suggest that adult hippocampal neurogenesis is influenced by enrichment from cell proliferation to cell survival, and maturation of the new neurones. In this research supporting data was observed from results of enrichment and in addition, data showing that the nature of neurones produced differ in structure, neuronal pattern and distribution between environmental groups in the dentate gyrus of the hippocampus of the Long-Evans rat. Also, a link exists between adult hippocampal neurogenesis and spatial learning as noted in the exercise environment where more cells were found on the ventral limb of the dentate gyrus when compared to the dorsal limb of the same rat in the exercise group.

Environmental enrichment increased brain weight. The brain weight was significantly increased ($p=0.05$) between the complex enriched environment (2.2 g) as compared to exercise and control environments (2.1 g and 2.0 g respectively) in the Long-Evans rat.

The body weight showed no significant difference ($p=0.05$) between groups in the Long-Evans rat. The mean weight of the control group was 467 g, the exercise 461 g and the complex enriched 455 g.

Synaptogenesis between the standard laboratory environment, exercise and the complex enriched environment showed no difference between groups in the Long-Evans rat.

Spinogenesis between the standard laboratory environment, exercise and the complex enriched environment showed no difference between groups.

The morphology of dendrite protrusions of the DCX immunopositive cells, shows category F post mitotic neurons evenly distributed on both limbs of the dentate gyrus of the hippocamus in the complex enriched environment, having more than one primary dendrite arising from the soma, and branching before reaching the molecular cell layer (complex structure and branching pattern). In contrast, neurons in the exercise environment were distributed mostly on the dorsal (spatial) limb, having more cells than the standard control environment with similar structure and branching patterns. Here, (the exercise and standard control groups) the category E post mototic neurones, had a single primary dendrite that extend to the MCL before branching into secondary and tertiary dendrites. The DCX immunopositive cells showed significant variation in the neuron number and neuronal arrangement on the dorsal and ventral limbs of the dentate gyrus between groups in the Long-Evans rat.

The DCX immunopositive cells showed a one-fold increase between the exercise and standard control environment, a three-fold increase between complex enriched and the exercise environment, and a four-fold increase between complex enriched and the standard control environment. There was no significant difference ($p=0.05$) observed in the DCX immunopositive cells in the dentate gyrus of the hippocampus of the Long-Evans rat as few number of animals were used.

The Ki-67 immunopositive cell count showed a three-fold increase between the exercise and standard control environment, a three-fold increase between the complex enriched and exercise environment, and a six-fold increase between the complex enriched and the standard control environment. There was no significant difference ($p=0.05$) observed, in the Ki-67 immunopositive cells in the dentate gyrus of the hippocampus of the Long-Evans rat as few number of animals were used.

Apoptotic cell numbers using pyknotic cell count showed a five-fold increase in the apoptotic cells between the standard control and exercise environment, a twenty-one-fold increase between the

exercise and complex enriched environment, and a twenty-seven-fold increase between the standard control and complex enriched environment. There was a significant difference (p=0.05) observed in the apoptotic cell count between groups in the dentate gyrus of the hippocampus of the Long-Evans rat, suggesting that proliferating cells unstimulated by activity did not differentiate and progress to maturity.

The volume of the dentate gyrus showed a fifteen-fold volume increase between the exercise and the standard control environment, a five-fold volume increase between the complex enriched and the exercise environment, and a nineteen-fold volume increase between the complex enriched and the standard control environments. There was no significant difference (p=0.05) in the volume of the dentate gyrus of the hippocampus of the Long-Evans rat.

Finally, various environmental factors as environmental enrichment and exercise have been related to spatial learning and memory, and shown to enhance adult hippocampal neurogenesis in the dentate gyrus of the hippocampus of the Long-Evans rat. Available data from adult hippocampal neurogenesis studies have contributed significantly to our understanding of the process of adult neurogenesis and its functions.

5.2 LIMITATION: The research was limited to the environmental enrichment paradigms only as the anticipated animals from the Rain forest and Desert were not subjected to the same techniques as the laboratory Long-Evans rats.

5.3 FUTURE RESEARCH

The outcomes of this research can be used to improve activity-based stimulation for special needs individuals, as well as in the aged people's homes with an attempt to infer on the benefits of adult hippocampal neurogenesis, as regards to its functionality in the brain and corellated to environmental enrichment on the overall brain function.

The outstanding component of this research work – the integrative pattern of the immature neurones will be carried on in a future research endeavour.

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