STRUCTURAL CHANGES OF HIPPOCAMPI AND EXPRESSION OF HSP70, C-FOS AND DREAM PROTEINS IN THE SPINAL CORD AFTER ACUTE THERMAL STIMULUS OF EARLY STRESS-EXPERIENCED RATS

by

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**Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy**

2014

ACKNOWLEDGEMENTS

Alhamdulillah, praise to Allah and His infinite mercy for giving me the opportunity to complete this thesis. I am deeply thankful to my dear supervisor, Prof. Dr. Zalina Ismail who gave me the chance to start and complete this study. I would also like to thank my co-supervisor Dr. Muhammad Taufik Hidayat for his strong support.

I am also highly appreciative of the assistance and moral support provided by the previous head of the Human Anatomy Department, Universiti Putra Malaysia, Dr. Mohamad Aris Mohd Moklas and my current head of Human Anatomy Department, Universiti Putra Malaysia, Prof. Dr. Fauziah Othman. Thanks to Dr. Che Badariah Abdul Aziz, the Head of Department of Physiology, School of Medical Sciences, Universiti Sains Malaysia (USM, for giving me opportunity to use the Physiology Laboratory). I am also grateful to Physiology Laboratory staffs for allowing me to run my experimental work there. To my fellow post-graduate students: Idris, Ija, Nizam, Aru and Lee, thanks for all your assistance.

To my families in Wakaf Che Yeh, Kota Bharu and Kampung Huma, Alor Setar, thanks for your support. To my son Danish Irfan and my daughters Damia Qistina and Darwisyah Qadriyyah, thanks for being patient and doing your homework together with mama. To my beloved husband Mr. Sobri, thanks for being my backbone and for giving me the support and strength to complete this study.

Finally, I would like to acknowledge Universiti Sains Malaysia for the financial support (Res. Uni Grant 1001/PPSK/812921) that was provided for completion of this study.

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Aβ	: Alpha beta
ABC	: Avidin-biotin peroxidase method
ACTH	: Adrenocorticotrophic hormone
AD	: Alzheimer's disease
Aδ	: Alpha delta
AMPA	: 2-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid
ANOVA	: Analysis of variance
AP-1	: Activator protein-1
ARASC	: Animal Research and Service Centre, Universiti Sains Malaysia
BDMA	: Benzyl dimentylamine
BGS	: Brain growth spurt
BrdU	: 5'- bromo-2'-deoxyuridine
BSA	: Bovine serum albumin
Ca^{2+}	: Calcium ion
$[Ca^{2+}]i$	: Intracellular calcium ion
CA1	: Cornu Ammonis-1
CA2	: Cornu Ammonis-2
CA3	: Cornu Ammonis-3
Ca/CRE	: Calcium/cAMP response element
CaM kinase	: Calcium-calmodulin dependent kinase
cAMP	: Cyclic adenosine monophosphate
CBP	: CREB-binding protein
CC	: Central canal
cdc2	: Cell division control 2

CGRP : Calcitonin gene-related peptide

CO : Corticosterone

COTH C : Corticosterone group without FST and received thermal stress

COTH F : Corticosterone group with FST and received thermal stress

COWTH C : Corticosterone group without FST and no thermal stress

COWTH F : Corticosterone group with FST and no thermal stress

CRE : Calcium-cyclic adenosine monophosphate response element

CREB : Cyclic adenosine monophosphate response element binding protein

αCREM : cAMP response-element modulator alpha

CRF : Corticotrophin-releasing factor

CTH C : Control group without FST and received thermal stress

CTH F : Control group with FST and received thermal stress

CWTH C : Control group without FST and no thermal stress

CWTH F : Control group with FST and no thermal stress

CS : Calsenilin protein

CVS : Cardiovascular system

DAB : Diaminobenzidine

dcs : dorsal corticospinal tract

DDSA : Dodecenyl succinic anhydride

DG : Dentate gyrus

dr : dorsal root spinal nerve

DLF : Dorsolateral fasciculus

DRE : Downstream regulatory element

DREAM : Downstream regulatory element antagonistic modulator protein

D-Pen3 : D-penicillamine3

D-Pen5 : D-penicillamine5

DPX : Diethyl(phenyl)xanthine

EC : Entorhinal cortex

EDTA : Ethylenediamine-tetra acetic acid

FLI : Fos-like immunoreactivity

ELISA : Enzyme linked immunosorbent assay

EMS : Early maternal separation

ER : Endoplasmic reticulum

FST : Forced swimming test

6'-GNTI : 6' Guanidinonaltrindole

GA : Golgi apparatus

GCL : Granule cell layer

GIT : Gastrointestinal tract

Gly-o1^{5} : Glycinol

gr : gracile fasciculus

GR : Glucocorticoid receptor

grp78 : glucose regulated protein 78

H_2O_2 : Hydrogen peroxidase

HPA : Hypothalamo-pituitary-adrenal axis

hrk : harakiri

HSC70 : Heat shock cognate 70

HSC73 : Heat shock cognate 73

HSE : Heat shock element

HSF : Heat shock factor

HSPs : Heat shock proteins

HSP27 : Heat shock protein 27

HSP40 : Heat shock protein 40

HSP60 : Heat shock protein 60

HSP70 : Heat shock protein 70

HSP70I : Heat Shock Protein 70-like immunoreactivity

HSP90 : Heat shock protein 90

HSP110 : Heat shock protein 110

ICER : Inducible cAMP early repressor

i.c.v. : intracerebroventricular

IEG : Immediate early gene

lfu : lateral funiculus spinal cord

IgG : Immunoglobulin G

IHC : Immunohistochemisry

IL-3 : Interleukin-3

IMM : Intermediomedial cell column

i.p. : intraperitoneal injection

KchIP3 : Potassium channel α subunit interacting protein

kDa : kilodalton

Kv4 : Shal-type (Kv4.x) K^+ channels are expressed in a variety of tissue, with particularly high levels in the brain and heart

LBDG : Lower blade of dentate gyrus

LCDs : Leucine charged domains

LPP : Lateral perforant pathway

LSp : Lateral spinal nucleus

MAPK : Mitogen-activated protein kinase

max : Maximum

MePh4 : Mephedrone4

mfp : mossy fibre

mGluRs : metabotrophic glutamate receptors

min : minute

ML : Molecular layer

MNA : Methylnadic anhydride

MPP : Medial perforant pathway

mRNA : messenger of ribonucleic acid

MR : Mineralocorticoid receptor

NA : Noradrenaline

Na^+ : Natrium ion

NaCl : Natrium chloride

NCS : Neuronal calcium sensor

NGF : Nerve growth factor

NGS : Normal goat serum

N/L : Neutrophil/lymphocyte ratio

NMDA : N-methyl-D-aspartate receptor

nor-BNI : Norbinaltorphimine

NRS : Normal rabbit serum

NS : Nociceptive specific

NSG : Non stress group

NTH C : nor-BNI group without FST and received thermal stress

NTH F : nor-BNI group with FST and received thermal stress

NWTH C : nor-BNI group without FST and no thermal stress

NWTH F : nor-BNI group with FST and no thermal stress

OD : Optical density

p72 : Also known as HSC70

PB : Phosphate buffer

PBS : Phosphate buffer saline

PC12 : Pheochromocytoma cell line 12

PFA : Paraformaldehyde

PKA : Protein kinase A

PKC : Protein kinase C

PNs : Projecting neurons

PND : Postnatal day

PVN : Paraventricular nucleus of the hypothalamus

Rat-2 cell : Is derived from a subclone of a 5'-bromo-deoxyuridine (BrdU)-resistant strain of the Fischer rat 3T3-like cell line RAT-1.

RER : Rough endoplasmic reticulum

RNA : Ribonucleic acid

RSG : Retrosplenial granule

RT : Room temperature

r-T3 : Reverse triiodotyronine

s.c. : subcutaneous

SDH : Sensory dorsal horn

SE : Standard error

SER : Smooth endoplasmic reticulum

SG : Stress group

SGZ : Subgranular zone

SP : Substance P

SPSS : Statistical package for the social sciences

SSC : Standard saline citrate

STT : Spinothalamic tract

SVZ : Subventricular zone

T3 : Triiodotyronine

T4 : Thyroxine

TBS : Tris base saline

TdT : Terminal deoxynucleotidyl transferase

TEM : Transmission electron microscope

TMB : Tetramethylbenzidine

TNZ : Thermoneutral zone

TUNEL : Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling

U-50,488H : trans-(±)-3,4-dichloro-N-methyl-N-[7-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide methane sulfonate

UBDG : Upper blade of dentate gyrus

Vfu : Ventral funicular spinal cord

VMnF : Ventral median fissure spinal cord

Vr : Ventral root spinal nerve

WDR : Wide dynamic range neurons

wk : week

ABSTRAK

PERUBAHAN STRUKTUR HIPOKAMPUS DAN EKSPRESI PROTEIN HSP70, C-FOS DAN DREAM DI KORDA SPINA SELEPAS RANGSANGAN KEPANASAN AKUT PADA TIKUS BERPENGALAMAN STRES

Kajian bertujuan untuk mengetahui kesan stres haba akut pada ekspresi protein HSP70, c-Fos and DREAM dalam usaha memahami mekanisma stres di peringkat neonat dan kesan pada kehidupan berikutnya. Fasa pertama kajian menilai ujian berenang secara paksa (FST) sebagai model induksi stres sederhana pada tikus neonat. FST dilakukan pada umur 7, 8 dan 9 hari selepas kelahiran. Kesemua tikus neonat hidup dan berada dalam keadaan sihat selepas FST. Berat badan mereka menurun pada hari ke 14 hingga 42 dan meningkat semula dan menyamai kumpulan kawalan pada hari ke 49 dan seterusnya. Nisbah sel neutrofil dan limfosit meningkat secara signifikan pada kumpulan FST berbanding dengan kumpulan kawalan. Skor BrdU menurun pada kumpulan FST berbanding tikus kawalan membuktikan berlakunya neurogenesis. Skor BrdU di bahagian atas dan bawah girus dentat adalah sama tetapi lebih tinggi berbanding kawasan CA1-3 hipokampus, talamus dan *retrospenial granular* (RSG). Skor BrdU di talamus dan RSG lebih rendah berbanding kawasan CA1-3 hipokampus. Ini menunjukkan bahawa FST adalah model yang baik untuk mengkaji stres akut dalam tikus neonat. Pada masa yang sama, model ini mampu menunjukkan perbezaan tindak balas stres di hipokampus.

Fasa kedua melibatkan kumpulan tikus dewasa yang mengalami FST semasa neonat (tikus berpengalaman stres) berbanding tikus tanpa pengalaman stres. Kedua-dua kumpulan dibahagi pula kepada kumpulan tikus kawalan (C), tikus yang menerima suntikan awal nor-BNI (N) dan tikus yang menerima suntikan awal kortikosteron (CO) sebelum didedah kepada stres haba pada suhu 42±1°C selama 15

minit. Dua jam berikutnya haiwan dimatikan, diikuti dengan penentuan ekspresi protein HSP70, c-Fos dan DREAM. Kematian sel neuron dinilai melalui kaedah imunohistokimia (IHC) dan mikroskop elektron transmisi (TEM). Pada kumpulan kawalan, HSP70 meningkat di kalangan tikus berpengalaman dengan stres dan haba (CTH F). Tanpa bersandar kepada pengalaman, ekspresi HSP70 adalah tertinggi pada kumpulan kortikosteron. Ekspresi c-Fos yang tertinggi adalah pada kumpulan kawalan tanpa pengalaman stres (CTH C) manakala kumpulan berpengalaman stres (CTH F dan COTH F) menunjukkan ekspresi c-Fos yang lebih rendah. Begitu juga, ekspresi DREAM yang tertinggi adalah di kalangan kumpulan kawalan (CWTH C dan CTH C) berserta COTH F, kemudian diikuti oleh CWTH F dan CTH F. Kesimpulannya, berbanding dengan tikus tanpa pengalaman stres, didapati bahawa tikus berpengalaman stres lebih berkemampuan menahan stres yang berikutnya. Pada kumpulan tikus nor-BNI, hampir kesemua protein kurang di ekspresi. Ini menunjukkan bahawa nor-BNI telah bertindak sebagai antagonis reseptor opiat kappa, yang mana menyebabkan tikus mengalami stres dan kesakitan yang minima. Kesimpulannya, pengalaman stres semasa neonat menyebabkan perubahan di sistem saraf periferi. Ini menunjukkan bahawa tikus berpengalaman stres lebih mampu menahan stres yang berikutnya semasa dewasa.

Namun demikian, kematian sel neuron masih berlaku di kawasan hipokampus CA3 diikuti dengan CA2 dan CA1 dan ini menunjukkan bahawa nor-BNI bertindak secara spesifik kepada tisu. Mikroskop elektron transmisi menunjukkan kemusnahan kepada sitoplasma, nukleus dan mitokondria manakala radas Golgi tidak mengalami sebarang perubahan. Tahap kemusnahan organel dan kematian sel adalah tertinggi pada kumpulan COTH F di mana kematian berlaku

sebaik sahaja diberi stres haba. Suhu rektum, walaupun tidak signifikan, telah menunjukkan peningkatan pada kesemua kumpulan kecuali pada COTH C yang mengalami hipotermia dan kemungkinan proses renjatan. Paras kortikosteron didapati tidak signifikan selepas dua jam stres haba, yang membuktikan bahawa paras telah menurun ke aras basal. Struktur dan fungsi hipokampus telah berubah akibat pengalaman stres semasa neonat dan ini menyebabkan pengurangan neurogenesis dan peningkatan kematian sel neuron. Kesimpulannya, stres haba akut pada tikus berpengalaman stres menyebabkan dua manifestasi yang berikut; (1) pada korda spina, stres haba akut dianggap "neuroprotective" dan kesan ini adalah khusus kepada tisu yang mengandungi reseptor opiat kappa di korda spina; (2) pada otak (terutama hipokampus), stres haba akut berupaya menyebabkan kematian sel neuron dan pengurangan neurogenesis.

ABSTRACT

STRUCTURAL CHANGES OF HIPPOCAMPI AND EXPRESSION OF HSP70, C-FOS AND DREAM PROTEINS IN THE SPINAL CORD AFTER ACUTE THERMAL STIMULUS OF EARLY STRESS-EXPERIENCED RATS

This current study was to explore the effect of acute thermal stress on the expression of HSP70, c-Fos and DREAM proteins in an attempt to understand the mechanisms of neonatal stress and the effect on stress later in life. Phase one of this study evaluated forced swimming test (FST) as a model for induction of moderate stress in neonatal rats. Forced swimming test was applied to neonatal rats on days 7, 8 and 9 of life. All the pups survived and remained healthy after FST. From day 14 to day 42, the FST group had significantly lower body weights when compared to the control group. Their body weights were back to control levels on day 49 onwards. Forced swimming test significantly increased neutrophil/lymphocyte ratios in the stress group compared to controls. There was downregulation of neurogenesis as evidenced by the decreased BrdU scores in the FST group when compared to controls. 5'- bromo-2'-deoxyuridine scores in both upper and lower blades of dentate gyrus were similar but higher than other subfields of hippocampus (CA1-3), thalamus and retrosplenial granular (RSG) areas. BrdU scores in the thalamus and RSG were significantly lower than that of the CA1-3 subfields of the hippocampus. Forced swimming test was found to be a good model for studying acute stress in neonatal pups. It was able to show differentiation of the stress response in the hippocampus.

Phase two involved the same group of rats subjected to FST in the neonatal period (stress-experienced rats) and included a comparison with non stress-

experienced rats. Both groups were subdivided into control (C), pretreatment with either nor-BNI (N) or corticosterone (CO) groups, before exposure to thermal stress at 42±1°C for 15 minutes. The animals were sacrificed two hours later, followed by determination of HSP70, c-Fos and DREAM proteins expression. Neuronal cell death was evaluated with immunohistochemistry (IHC) and TEM respectively. In the control group, HSP70 was upregulated in stress-experienced and thermal rats (CTH F). Regardless of experience, the corticosterone group had the highest expression of HSP70. The highest expression of c-Fos was in the stress-experienced control group (CTH C) while stress-experienced groups (CTH F and COTH F) showed lower levels of c-Fos expression. Similarly, the highest expression of DREAM protein was in the control groups (CWTH C and CTH C) and COTH F, followed by CWTH F and CTH F. Thus, stress-experienced rats were better able to withstand subsequent stress compared to rats with no previous stress experience. In the nor-BNI group almost all proteins were less expressed, showing it worked as a kappa opioid antagonist, where the animals experienced less pain and minimum stress Thus, neonatal stress experience caused changes in the peripheral nervous system indicating that stress-experienced rats are better able to withstand subsequent acute stress during the adult stage.

However, neuronal cell death remained present in the hippocampus in CA3, followed by CA2 and CA1 subfields, suggesting that nor-BNI was tissue specific. Transmission electron microscope showed damaged cytoplasm, nucleus and mitochondria, whereas the Golgi apparatus was unaffected. COTH F group has severe damage of organelles and death occurred immediately after heat stress. Rectal temperature showed an incremental pattern even though it was not significant except

for COTH C where there was hypothermia and probably shock. Corticosterone level was not significant after two hours thermal stress suggesting it had returned to basal levels. The structure and function of the hippocampus was altered by neonatal stress experience resulting in depression of neurogenesis and increased neuronal cell death. Thus, acute thermal stress in stress-experienced rats result in a dual manifestation: (1) in the spinal cord, the acute thermal stress appears to be neuroprotective and this effect is tissue specific to the kappa-opioid receptors in the spinal cord; (2) in the brain (especially), acute thermal stress appears to cause persistent neuronal cell death and depression of neurogenesis.

CHAPTER 1

INTRODUCTION

1.1 THEORY AND CONCEPT OF STRESS

Selye (1976), a pioneer in stress research, defined stress as "the nonspecific response of the body to any demands made upon it". These demands are a result of various factors that disrupt homeostasis and are collectively known as stressors. These factors increase the demand for readjustment (Selye, 1974) and lead to an overall disruption of body responses that is defined as stress. As a result, the body is forced to make adaptive changes via various non-specific responses in order to maintain homeostasis.

A similar definition can be found in Bailliere's Comprehensive Veterinary Dictionary (1988) where stress is defined as the sum of biological reactions to any stimulus, which tends to disrupt the homeostasis of organisms. In general, biologists define stress "as a physiological reaction involving heightened activity of the pituitary and adrenal cortex (Hill, 1983) in order to maintain homeostasis". Stress can also be observed within the context of veterinary science and agriculture where conditions such as climate change, social interactions, stock density, nutrition, disease and human interactions (Harvey *et al.*, 1984) are examples of potential factors that may affect homeostasis and therefore result in stress of an animal. Thus, interestingly, while all definitions of stress are related to the disruption of homeostasis, nevertheless, as pointed out by the American Institute of Stress (http://www.stress.org/), there is no actual single and specific definition of stress as what is stressful for one person or animal may be pleasurable or have little effect on

others. Animals and people all react to stress in different ways and this makes it very difficult to experimentally design research paradigms that specifically address the concept of stress. In addition, any experimental design involving stress is further complicated by the fact that there are numerous factors, which may appear harmless but have the potential to be stressors. These factors may be in the form of physical (heat, noise), chemical (food, hormones), microbiological (viruses, bacteria or parasites), physiological (tumours, abnormal function), developmental (old age, genetic changes) or psychological (emotional and mental disturbances) stimuli (van Wynsberghe *et al*., 1995).

Stress can be deleterious especially if it is in combination with injury, pain or disease. However, it must be realised that while stress may be damaging to biological systems, nevertheless, it does have a positive effect of enabling the organism to adapt to change and maintain homeostasis in the presence of rapidly changing situations. Thus, stress may lead either to a positive or negative outcome, depending on the interactions between individual characteristics and the properties of stressors, stress and physiological systems of the organism (Carlson, 1994).

1.2 STRESS MODELS

As mentioned above, stress is a multi-factorial and multi-dimensional concept that can be represented by an equally varied number of stress models. Recent studies on stress have used many different models that are applicable for use in adult laboratory animals. These stress models include animal restraint (Bain *et al*., 2004), exposure to a novel environment (Tang & Verstynen, 2002), cold and warm temperatures (McKitrick, 2000), feed restriction (Zulkifli *et al*., 2002) and sleep

deprivation (Mirescu *et al*., 2006). While these are well-established models of stress in adult animals, nevertheless, a suitable stress model in neonatal laboratory animals is still not well established.

There are several stress models that have been used in neonatal laboratory animals, such as early postnatal handling (which causes prolonged anxiety) as described by Plotsky and Meaney (1993), maternal separation (which causes exaggerated hormonal responses and altered neurotransmitter release) (Ladd *et al*., 1996) and feed restriction (Zulkifli *et al.*, 2002). Nevertheless, early postnatal handling is not easy due to the difficulty in maintaining consistency of handling. Similarly, Zulkifli *et al*. (2002) showed that moderate stress could be induced early in life by moderate feed restriction (60% at 4, 5 and 6 days of age) resulting in heat tolerance later in life through enhanced heat shock protein 70 (HSP70) response. However, the procedure is quite tedious and a lot of time is required for measuring food and body weight in order to calculate the actual amount of food that was needed by the animal for each day of feed restriction. The various disadvantages of these models of moderate stress were the main reason why there is still a lack of proper stress models in neonatal animals. In this current study, the moderate stress was achieved not through feed restriction but through forced swimming test (FST). The procedure is simple, low-cost, easy to manage and has proven its reliability across laboratories for testing potential antidepressant activities (Slattery & Cryan, 2012). Therefore, this study evaluated FST as a model for creating a moderate stress situation in neonatal rats. This current study also evaluated the degree of tolerance of the pups to the FST model, which mimics the watery medium and floating sensation inside the uterus during the prenatal period.

1.2.1 Forced swimming test

The FST was first used in rats and mice by Porsolt *et al*. (1977). Since then, it has been widely accepted as a model for the measurement of the effects of antidepressant treatment especially in adult animals (Cryan *et al*., 2005). However, this stress model has not been used in neonatal rats. Therefore, this current study evaluated the effectiveness of FST to induce stress in neonatal rats. The effect of stress was measured through neutrophil/lymphocyte ratios and weekly body weight. Neurogenesis was also evaluated to see the significance of stress experience early in life on the development of the hippocampus.

1.3 EFFECT OF STRESS EXPERIENCE IN EARLY LIFE

Stress is a universal phenomenon in both humans as well as animals and has been extensively studied in various contexts. However, despite the large body of knowledge on stress (Shalev *et al*., 2000; Koolhaas *et al*., 2011) it is as yet unclear as to the mechanisms involved in the effect of stress during the neonatal period of life.

Interestingly, it has been shown that handling of laboratory animals during their first few weeks after birth, inclusive of a brief separation from their mothers, was found to decrease age-related learning disturbances and increased resistance to the effects of later stressors (Meaney *et al*., 1996). It has also been shown that animals which experienced stressful stimulation during the first 21 days of life showed basal concentrations of adrenocortiotrophic (ACTH) and corticosterone comparable to that of non-stimulated animals. However, as adults, when exposed to a stressor, the stimulated animals displayed blunted ACTH and corticosterone responses and a faster return to basal hormone levels. It is postulated that these

changes involve complex neuronal changes associated with dysfunction of the hypothalamo-pituitary axis (HPA) (Meaney *et al*., 1996) while other researchers found a link in relation to the propensity to consume alcohol during later adulthood (Jones *et al*., 1985; Lancaster, 1998).

Liu and colleagues (1997) conducted studies to determine why brief handling involving separation from the mother had such pronounced and persistent effects. After reuniting with their young following the brief separation, mothers exhibited increased licking, grooming, and nursing of their offspring. The researchers correlated these maternal responses with altered hormonal responses to stressors. They also suggested that maternal behavioural style acted to "programme" hypothalamo-pituitary-adrenal (HPA) responses to later environmental stressors, perhaps including factors like alcohol intake. However, the actual mechanisms involved in changing the HPA responses were not clarified.

Anisman *et al*. (1998) studied two mouse strains that exhibit very different behavioural and neurochemical profiles in response to stressors. The more stress-reactive strain displayed relatively poor maternal behaviour, compared to the less stress-reactive strain (Anisman *et al*., 1998). However, when young mice of the stress-reactive strain were raised by mothers from the less reactive strain (cross-fostered on the day of birth), some behavioural disturbances and the exaggerated HPA alterations of the more reactive mice were decreased. However, maternal behaviour alone is not sufficient for this outcome to emerge because it has been shown that being raised by a mother from the more reactive strain did not result in behavioural or hormonal disturbances in young mice of the more resilient strain.

Thus, it appears that heightened stress reactivity in these mice result not only from inadequate maternal care but also a combination of genetic factors (Zaharia *et al*., 1996). In this present study, the FST model was used to examine the expression of several stress-associated molecular markers in an attempt to evaluate the molecular mechanisms associated with stress in the neonatal period. Indeed, this current study focused not only on the effect of early neonatal stress but also examined if this early stress has any positive influence on the ability to tolerate subsequent stress later in life.

1.4 NEUROGENESIS

Neurogenesis is most active during pre-natal development where cells proliferate, survive and differentiate into neurons. Neurogenesis can continue throughout adulthood predominantly in two regions (Eriksso*n et al*., 1998):

- The subventricular zone (SVZ) lining the lateral ventricles, where the new cells migrate to the olfactory bulb via the rostral migratory stream.
- The subgranular zone (SGZ), part of the dentate gyrus of hippocampus.

The formation of new neurons can be divided into three major steps (Andersson, 2010) and in the hippocampus, as shown in Figure 1.1, the three steps include: proliferation of a neuronal stem cell in the SGZ, migration into deeper granular cell layers (GCL) and differentiation, where the mature neuron send out dendrites into the molecular layer (ML) and the axon (mossy fibre) through the hilus to the CA3 field.

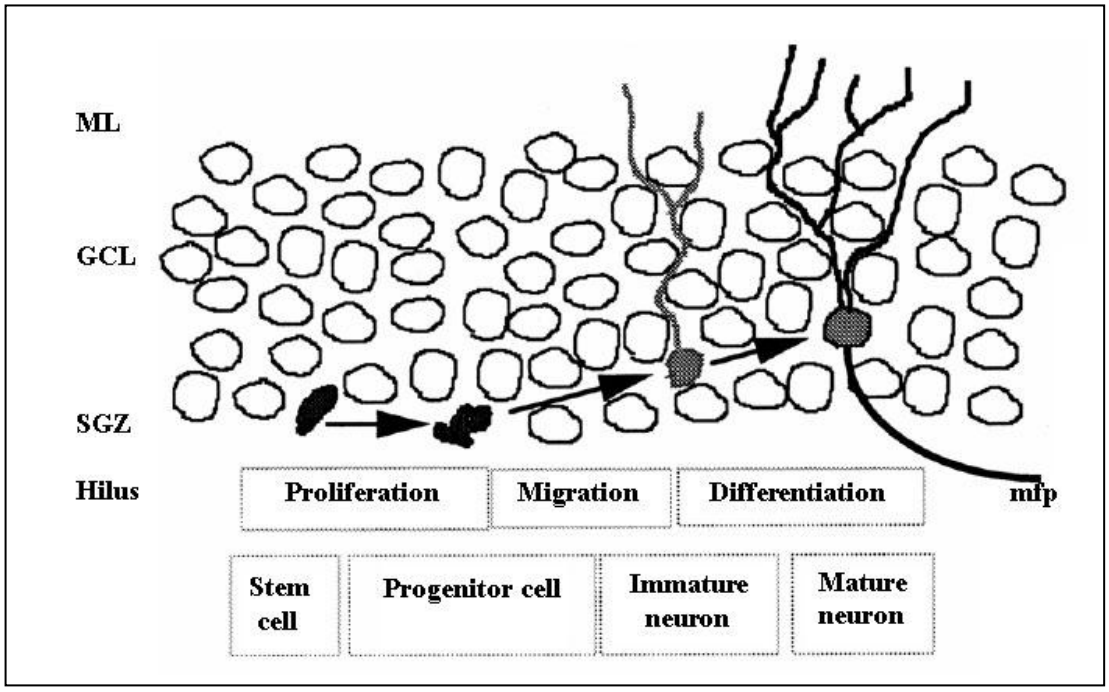

Figure 1.1: Neurogenesis in the dentate gyrus (DG).

ML – molecular layer, GCL – granule cell layer, SGZ – subgranular zone and mfp – mossy fibre.

Adapted from Aberg (2007).

In the adult brain of rodents (Markakis & Gage, 1999), non-human primates (Kornack & Rakic, 1999; 2001), and humans (Eriksson *et al*., 1998), neurogenesis appears to occur throughout life in the anterior part of subventricular zone of the lateral ventricle and the GCL of the hippocampus. Here, the cells arise from progenitors within the border of the hilus and DG and accumulate in the DG (Seaberg & van der Kooy, 2002). Newly generated neurons in the DG are morphologically distinguishable from other granule cell neurons (van Praag *et al*., 2002), may be long lived, may contact and receive appropriate targets from the existing hippocampal circuitry, generate action potentials and have functional synaptic inputs and may be important for learning and/or memory formation (Shors *et al*., 2001).

Several different factors that regulate neurogenesis have been identified. It has been shown that physical activity and environmental conditions can affect proliferation and survival of neurons in vertebrates (Kempermann & Gage, 1999) as well as invertebrates (Cayre *et al*., 1996). It has also been found that crayfish in an "enriched" environment (filled with gravel, foliage, tunnels, a tree-like structure, and a rock) developed increased neurogenesis and neuronal survival compared to siblings in an "impoverished" environment (Ayub *et al*., 2011) Hormones have also been found to influence the rate of neurogenesis in vertebrates (e.g. testosterone) and invertebrates (e.g. ecdysone). Serotonin is believed to play a key role in neurogenesis in a variety of organisms (Beltz *et al*., 2001). In lobsters, depletion of serotonin dramatically reduced the proliferation and survival of olfactory projection neurons (Beltz *et al*., 2001) and local interneurons (Benton & Beltz, 2001). Most recently, neurogenesis was found to follow a circadian rhythm in the juvenile lobster (Goergen *et al*., 2002) where significantly more neurons were formed at dusk, the most active time for lobsters, than at any other time of the day. Thus, in this present study, the

presence of neurogenesis is an important indicator of increased stimulation, increased hormone release and greater physical activity: all of which occur in the presence of stress. In the context of this study, the focus will be on acute thermal stress.

1.5 THERMAL STRESS

Body temperature represents the balance between heat production and heat loss and it is controlled by the thermoregulatory centre in the hypothalamus. Therefore, when animals are exposed to thermal stress, the heat loss centre will be activated to protect the body from excessively high temperature, which can be particularly damaging to the body. Most heat loss occurs through the skin via the physical mechanisms of heat exchange – radiation, conduction, convection and evaporation. Heat loss centre triggers one or both of the following: 1) vasodilation of cutaneous blood vessels and 2) enhanced sweating. If normal heat loss processes become ineffective, the hyperthermia that ensues depresses the hypothalamus. As a result, heat-control mechanism suspended, creating a vicious positive-feedback cycle. Sharply increasing temperatures increase the metabolic rate, which in turn increased the heat production. The skin becomes hot and dry and as the temperature continues to spiral upward, multiple organ (including brain) damage becomes a distinct possibility. This condition, called heat stroke, can be fatal (Gomes Ramos *et al*., 2012).

The thermoneutral zone (TNZ) is the temperature tolerance range of the body where there is a balance between heat gain and loss in order to ensure comfort (Hafez, 1968). The organism adjusts to the temperatures within the zone through different responses requiring little energy. However, heat stress, as indicated by elevated body temperature, occurs when environmental extremes, either acute or

chronic, lead to alterations in the rate of heat production and a rise in body temperature (Francis *et al*., 1991). This increase in body temperature (hyperthermia) as a response to thermal exposure is a well-established mechanism (Ahmed *et al*., 2007). It can often be attributed to failure of the proper physiological and behavioural responses of the peripheral receptors, hypothalamus, nervous system, endocrine glands or enzymes thus leading to heat stress.

1.5.1 Physiological responses to heat stress

A variety of physiological responses are evoked to cope with changes in ambient temperatures (Harvey *et al*., 1984). In birds, blood flow is diverted from certain internal body organs such as the liver, kidneys and intestines to dilated blood vessels of the peripheral tissue (skin) in order to facilitate heat loss (Darre & Harison, 1987). The lungs and kidneys along with various buffers systems play an important role in preventing rapid changes in the blood pH. However, as the respiratory rate increases in heat-stressed broilers, there is a corresponding decrease in the levels of blood carbon dioxide. These changes may alter the acid base balance and subsequently lead to hypocapnia and respiratory alkalosis (Teeter & Smith, 1986).

Exposure of humans or animals to high ambient temperatures poses several problems. In the poultry industry it may result in a reduction of egg production, egg size, feed consumption, feed efficiency, growth rate, hatchability and survivability (Kuttlu & Forbes, 1993). While in humans, the primary signs and symptoms of heat stroke are confusion, irrational behaviour, loss of consciousness, convulsions, a lack of sweating (usually); hot, dry skin, and an abnormally high body temperature, e.g., a

rectal temperature of 41°C. If body temperature is too high, it can be fatal **(**Che Norma, 2000).

Similarly, heat stress can retard the growth rate of broilers when they are reared at temperatures higher than 21 to 25°C (Meltzer, 1980). Modern fast growing broiler chicks must consume large quantities of feed in order to attain maximal growth rate. However, the intake and metabolism of feed have a thermoregulatory effect and at high environmental temperatures the heat increment aggravates the problem by adding more heat to an already heat stressed system (Kuttlu & Forbes, 1993). The birds, therefore react by reducing voluntary feed intake (Howlider & Rose, 1987) and metabolic rate, resulting not only in poor feed efficiency and body growth (van Kampen, 1981) but also in decreased egg production (Clark & Sarakoon, 1967). Geraert *et al*. (1996) indicated that half of the growth reduction in hot environments was due to a direct effect of high temperature. This reduction of efficiency was partly explained by decreased metabolic utilisation of nutrients, increased heat production, reduced protein retention, and enhanced lipid deposition (Ain Baziz *et al*., 1996).

1.5.2 Biochemical responses to heat stress

In animals, aversive stimuli such as heat stress, can lead to an imbalance of various biochemical substances in blood (Teeter *et al*., 1985). However, the literature on the effects of heat stress on biochemical reactions is often contradictory. In the face of an acute stressor, the blood glucose level increases (Collier *et al*., 1982), and Webster (1976) attributed the phenomenon to depression of both catabolic and anabolic enzyme secretions, retarded glucose utilisation and consequently decreased

metabolic rate. Another explanation from Thompson, (1973), linked the hyperventilation of acute stress to the increase in level of glucocorticoid hormone which consequently led to an increased breakdown of glycogen into glucose. Incontrast, studies in ruminants (Alnaimy *et al*., 1992) demonstrated that blood glucose level decreased significantly in response to high ambient temperature. This could be due to the marked dilution of blood and body fluids as a whole in the heat stressed animals (Habeeb, 1987) on the increase in glucose utilisation to produce more energy for greater muscular activity required for high respiratory response. On top of that, the reduction in production of propionic acid in the rumen and the decrease in feed intake as well as hepatic capacity for gluconeogenesis (Sano *et al*., 1983) could also be linked to lower plasma glucose levels in heat stressed animals. Exposing chickens to a temperature of 41°C may increase body temperature to between 44.5°C to 45.0°C with an associated increase in plasma sodium and chloride and a concurrent decrease in plasma potassium and phosphate (Ait-Boulahsen *et al*., 1989). In normally hydrated fowls however, heat stress (35°C to 45°C for 10 to 12 hours) produced no significant changes in the serum concentrations of chloride, potassium, sodium and calcium, or in the serum osmolarity, although serum phosphate levels declined (Arad *et al*., 1983).

1.5.3 Hormonal responses to heat stress

The Merriam-Webster's Medical Dictionary (http://www.merriam-webster.com/) defines hormones as chemical substances, which are formed in one organ or part of the body and carried in the blood to another organ or part where they exert functional effects. Hormones can modify the functional activity, and sometimes

alter the structure, of just one organ or tissue or various numbers of them. They are produced and release based on the physiological demands-of the body system.

1.5.3 (a) Corticosteroids

When an animal is exposed to a stressor, the paraventricular nucleus of the hypothalamus (PVN) releases corticotrophin-releasing factor (CRF) which stimulates the anterior pituitary gland to secrete ACTH (Varghese & Brown, 2001). ACTH enters the general circulation and stimulates the adrenal cortex to synthesise and release corticosteroids. At the cellular level, ACTH alters the production of cellular proteins and enzymes (Gross & Siegel, 1993). In conditions of stress, increased levels of corticosterone promote gluconeogenesis from muscle proteins and lipolysis of adipose tissue for immediate energy requirements. However, prolonged hypersecretion of corticosteroids may result in cardiovascular disease, hypercholesterolemia, gastrointestinal lesions, retardation in growth, reduced reproductive capability (Moberg, 1985) and immunosuppression (Roth, 1985).

1.5.3 (b) Thyroid hormones

The importance of the thyroid gland in adaptation to heat stress is related to the central role that thyroid hormones play in the regulation of metabolism (McNabb, 1988). In chickens, thyroid hormone secretion is depressed as environmental temperatures increase, thus heat tolerance improves as thyroid function is reduced. The two active forms of thyroid hormones are thyroxine (T4) and triiodotyronine (T3) while the inactive form is reverse triiodotyronine (r-T3). When animals are exposed to warm temperatures, T4 is activated by conversion into r-T3, whereas during cold exposure T4 is converted into T3, which stimulates metabolic rate.

Prolonged exposure of animals to high temperatures decreased T3 but not T4 concentrations. The explanation for the phenomenon has yet to be documented.

1.5.3 (c) Aldosterone

Aldosterone, a steroid hormone secreted by the adrenal cortex, causes sodium retention. Aldosterone hormones are known to play an important role in controlling body fluid together with vasopressin hormones. In cattle, plasma aldosterone concentration has been reported to decrease following heat stress (Niles *et al*., 1980). This could be attributed to a large decrease in potassium retention in heat stress (Kamal *et al*., 1962). With prolonged heat stress, mineralocorticoids seem to decrease due to the change in blood electrolytes. The increase in body fluids which occurs in heat stressed cattle may be partly responsible for this decrease (Alnaimy *et al*., 1992), since the increase in the extracellular fluid volume decreases the aldosterone secretion.

1.5.3 (d) Catecholamines

The catecholamines are secreted by specialised cells derived from the neural crest and located in the medulla of the adrenal gland. These hormones can be divided into two types: adrenaline (A) and noradrenaline (NA) which are synthesised and released from the adrenal chromaffin cells. Harvey *et al*. (1986) indicated that, the response of both hormones to stress is similar to that of corticosterone since both adrenal cortical hormone and ACTH stimulate the release of both A and NA. Both adrenal catecholamines have a large number of actions, most of which contribute to the sympathetic fight-or-flight response. They promote glycogenolysis (breakdown of glycogen to glucose-1-phosphate) in skeletal and cardiac muscles. This action

mobilises glucose in those tissues (e.g. muscle and cardiovascular system tissues) that are typically involved in response to emergencies. In addition, these hormones stimulate the strength and rate of the heart beat and the contraction of vascular smooth muscle, thereby raising the blood pressure (Kadetoff, 2012).

1.5.3 (e) Melatonin

Melatonin is an indolamine hormone synthesised and released by the pineal body during the hours of darkness. Melatonin has been implicated in thermoregulation in birds (John & George, 1991) and may regulate the circadian rhythm in body temperature. John *et al*. (1978) indicated that in pigeons, body temperature is relatively lower in the night when both plasma and pineal levels of the melatonin are high. In contrast, body temperature is higher in the day when the melatonin levels are low. High concentration of melatonin hormones may help the animal to dissipate heat by enhanced process of vasodilation and blood flow to peripheral tissue particularly to the foot (Jones & Johansen, 1972). In addition, melatonin also acts centrally by lowering the set point of the main thermostat, which is believed to be present in the hypothalamus (John & George, 1991).

1.5.4 Feed and water intake

High ambient temperatures stimulate the peripheral thermal receptors to transmit suppressive nerve impulses to the appetite centre in the hypothalamus causing reduced feed consumption (Alnaimy *et al*., 1992). Thus, less nutrients are available for enzymatic activities, hormone synthesis and heat dissemination, which minimises thermal load (Kamal, 1975). In mammals, exposure to severe heat, suppresses the production of hormone releasing factor by the hypothalamus, causing

a decrease in pituitary hormonal secretion (Johnson, 1974), insulin and possibly thyroxine (Habeeb, 1987). According to Niles *et al*. (1980) these changes may slow down the metabolic pathways, causing drastic impairment of protein utilisation. Under these situations, the rate of protein synthesis is unable to compensate for the increase in protein metabolism, which leads to a negative nitrogen balance. The destruction in protein tissue is due to an increase in glucocorticoid hormone which is responsible for protein catabolism (Selye, 1976).

Increasing environment temperature may change the water intake regime, where animals will consume more water (Deyhim & Teeter, 1991). The increase in water consumption occurs immediately (May & Lott, 1992), in order to balance for water loss through evaporative cooling (Mench, 1985). The intermediate increase in water consumption meets the intermediate demands of evaporative cooling from respiratory surfaces and associated decline in food consumption reduces the contribution of metabolic heat to the total heat load that requires dispersion.

1.6 THE CELLULAR STRUCTURE

A brief description of the cell is provided as background for the explanation regarding changes in the cell following heat stress. Basically, a cell (Figures 1.2 and 1.3) has a nucleus, which is surrounded by cytoplasm. The nucleus has a nuclear membrane, chromatins and a nucleolus. It also contains important genetic material within the chromosomes. The cell membrane not only provides the structure and shape, but it is protective in function as the selectively permeable cell membrane, controls movement of materials across the cell membrane.

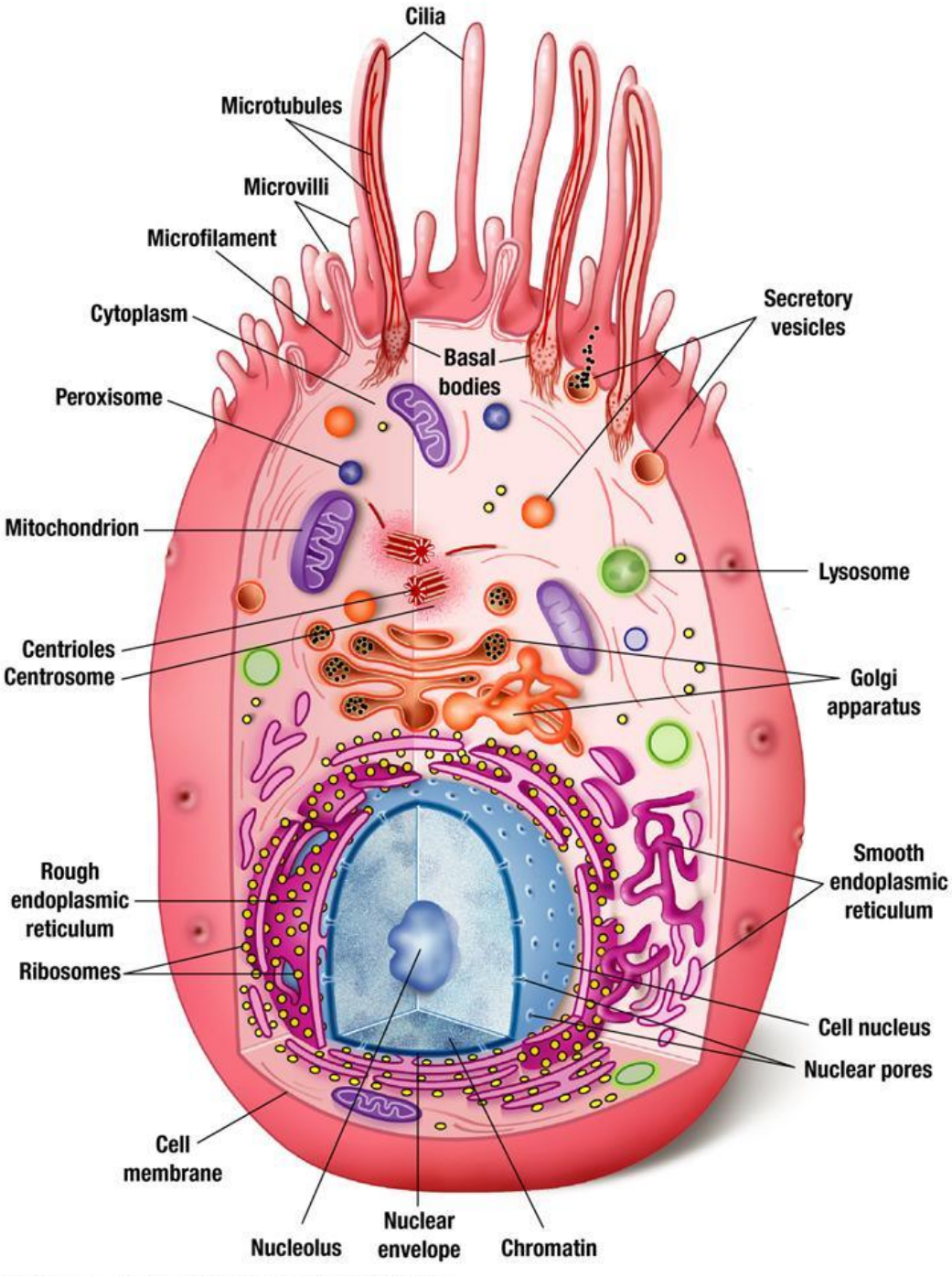

Figure 1.2: A eukaryotic cell, its cytoplasm, and its organelles.

Adapted from Eroschenko (2008).

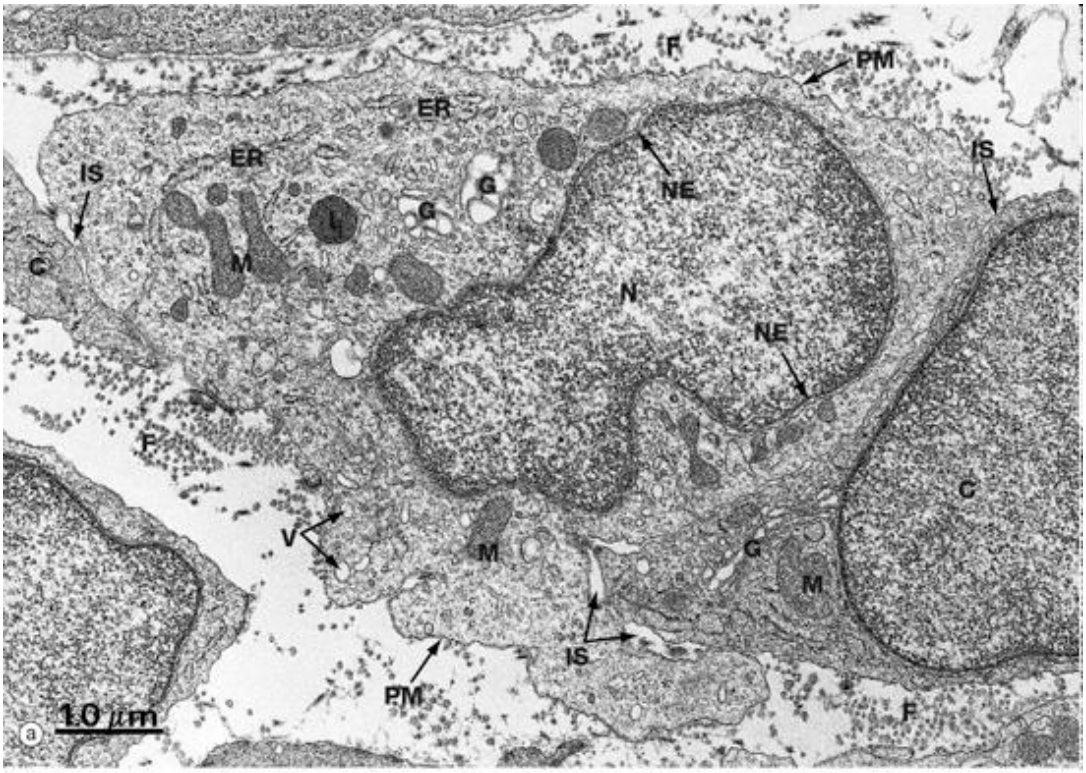

Figure 1.3: Micrograph of a eukaryote cell consists of organelles such as cytoplasm (C), endoplasmic reticulum (ER), golgi apparatus (G), lysosomes (L), mitochondrial (M), nucleus envelope (NE), plasma membrane (PM) and Vacuoles (V).

Adapted from Eroschenko (2008).

The endoplasmic reticulum (ER) transports material from one part to another part of the cell. There are two types of endoplasmic reticulum:

- RER is the rough endoplasmic reticulum (attached to ribosomes)
- SER is the smooth endoplasmic reticulum (no ribosomes)

The ribosome is made of ribonucleic acid (RNA) and protein enzymes and it has an important role in protein synthesis. Mitochondria are known as the "powerhouses" of cell. They consist of a double-layered membrane where the inner part consists of folds, called cristae that assist in the breakdown of glucose molecules. The energy released through this process is stored in the form of adenosine triphosphate (ATP). The Golgi apparatus is made up of numerous layers which form sac-like structures and help in protein packaging and its distribution to other parts of the cell.

Centrioles lie near the nucleus and are made up of nine tube-like structures, each of which has three tubules. They release spindle fibres which attach to chromosomes during the cell division. Lysosome is a structure containing several enzymes. It helps for the breakdown of larger molecules into small parts and is also responsible for the transport of waste out of the cell. Vacuoles store food and water and provide turgor pressure against the cell walls.

1.6.1 Structural changes due to heat stress

1.6.1 (a) Concepts in cell injury

When animals or humans are exposed to any form of stress, their body system will try to restore homeostasis. Failure of homeostasis results in cell injury.

Cell injury can be divided into two types: reversible and irreversible injury. In reversible injury, the cell is able to overcome the insult, while irreversible injury can eventually lead to cell death (Figure 1.4). Cell death can be classified into two major types: necrosis and apoptosis.

i. Necrosis

Necrosis is characterised from the activities of diseased organisms, toxins or physical factors, as well as inadequate nutrition or starvation due to an interrupted normal blood flow or avascular necrosis (Bejar *et al.*, 2005). Changes of morphologies in cells undergoing necrosis result from enzymatic degradation and denaturation of proteins of cellular components (Majno & Jovis, 2004). Those tissues having necrosis may undergo characteristic alterations over time. Grossly, all the necrotic tissues undergo colour changes and acquire a firm consistency within the first 24 hours except for the brain. Within two to three days, a mark delineated by an inflammatory reaction is presented, which may have fibrinous exudates. Then, a gray/white periphery area, due to healing process, occurs after a week, and after several months, a fibrous scar develops. However, in brain tissue the gross changes start with a softening and loss of tissue definition resulting in extreme softening after two to three days and a rim of peripheral hyperaemia. After several months, a cystic area traversed by fibrous strands may be seen.

Microscopically, the earliest changes include a mild degree of cytoplasmic oedema, dilatation of endoplasmic reticulum, slight mitochondrial swelling, disaggregation of polysomes and the present of small aggregates of condensed

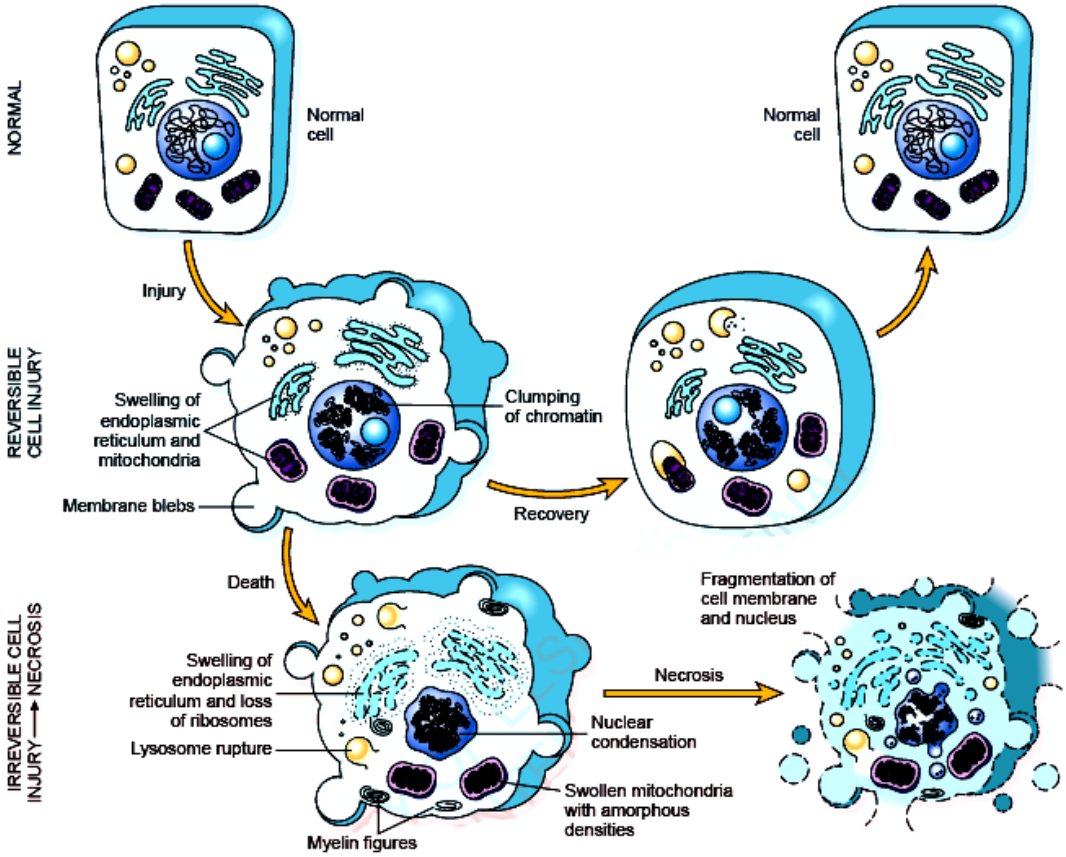

Figure 1.4: Schematic diagram of normal cells and the changes in reversible and irreversible cell injury.

Adapted from Mitchell and Cotran (2003).

chromatin around the nuclear periphery (Mitchell & Cotran, 2003). All the alterations are probably all reversible, but in dying cells are followed by a further set of changes that are irreversible; it is the stimulation of these which defines the 'point of return' in the route to death (Laiho & Trump, 1975).This second set includes 'high amplitudes' swelling of mitochondria – a florid dilation with rupture of internal cristae and usually, development of matrix densities of flocculent or granular types. Later lethal alterations include extensive cytoplasmic swelling, dissolution of cytoplasmic organelles and rupture of plasma membranes.

Necrosis can be classified into three types: coagulation necrosis occurs as a result of protein denaturation (cell shape and tissue architecture are maintained remained and it is the most common type of necrosis), colliquative or liquefactive necrosis results from enzymatic degradation (does not involve the preservation of tissue architecture), and fat necrosis is due to the action of fat degrading enzymes known as lipases and occurs in tissue with a high fat content such as pancreas (Brauchle, *et al*., 2014).

ii. Apoptosis

Apoptosis has been observed in a variety of tissue including; prostate, adrenal cortex, endometrium, thymus and embryonic tissue. Apoptosis, by contrast, is inherently "programmed" as part of cellular processes, allowing the cell to die in response to variety of signals without seriously affecting neighbouring cells, that is it does not elicit an immune response. It is frequently physiological, and regulated by changes in the levels of recognised trophic hormones or by lesser known but undoubtedly physiological factors in embryonic development. Further, necrosis

apparently represents loss of plasma membrane volume control, initiated, or at least perpetuated by collapse of cellular energy supply, but the initiation of apoptosis in several different types has been shown to require continuing macromolecular synthesis and perhaps new gene activation and involve the early appearance of non-lysosomal nuclease activity in the nucleus. Apoptosis is a common and essential form of cell death that occurs under both physiological and pathological conditions such as the apoptosis present during development of the hands and tail of frogs (Mosser & Martin, 1992).

Details of the morphology of apoptosis have been extensively reviewed (Asadi-Shekaari *et al*., 2009). Ultrastructurally the earliest changes of apoptosis include the loss of cell junctions and other specialised plasma membrane structures such as microvilli. At the same time the cytoplasm becomes condensed and nuclear chromatin marginates into one or several large masses, which initially may blister the nuclear membrane outwards and then coalesce to form crescentic caps around half or more of the nucleus. Apoptosis involves separation of the cell from it neighbours, condensing of the cytoplasm, condensing of the plasma membrane and finally blebbing off apoptotic bodies, which contain various organelles and chromatin fragments (Figure 1.5). In such organisms, total cell number is a function of both cell proliferations via mitosis and cell death. Necrosis is rarely seen under physiological conditions. It is almost always associated with an inflammatory response and neighbouring cell damage (Cotter & Al-Rubeai, 1995). Such an event would obviously have detrimental effects on the whole organism. This is unlike apoptotic cell death where the contents of dying cells remain within sealed vesicles until the apoptotic cell is removed through phagocytosis.

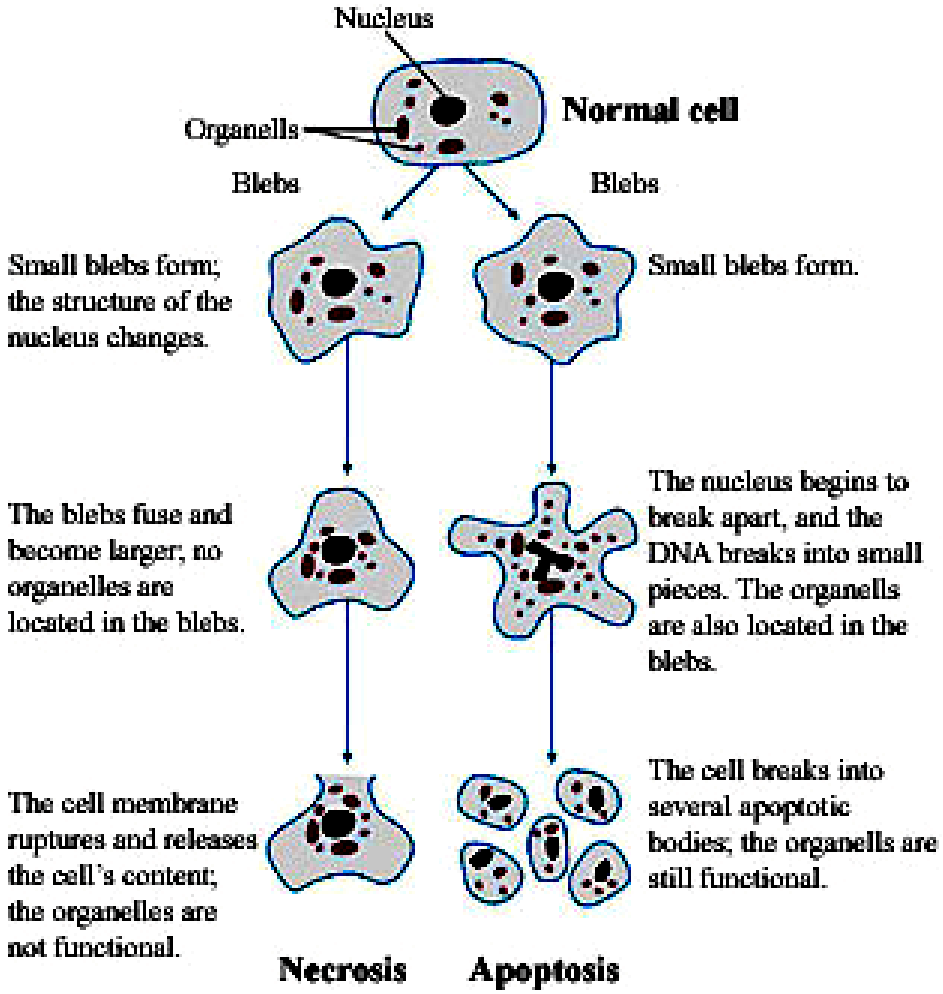

Figure 1.5: Differences between necrosis and apoptosis.

Adapted from Mitchell and Cotran (2003).