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United Arab Emirates University

College of Medicine and Health Sciences

Department of Pharmacology

SHORT-TERM BILATERAL ADRENALECTOMY: BIOCHEMICAL AND MORPHOLOGICAL ALTERATIONS IN THE RAT HIPPOCAMPUS

Ahlam Said Abi Issa

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

Under the Supervision of Professor Abdu Adem

April 2017

Declaration of Original Work

I, Ahlam Said Abi Issa, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Short-Term Bilateral Adrenalectomy: Biochemical and Morphological Alterations in the Rat Hippocampus*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Abdu Adem, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: Date: 22/5/17

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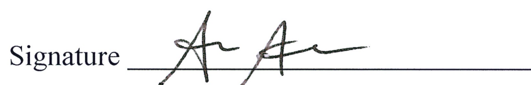
   Signature  Date 1/5/2017

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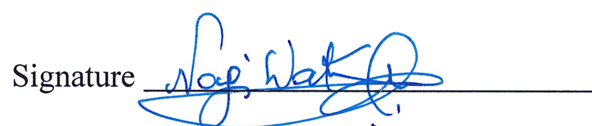
Signature  Date 22/5/2017

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Abstract

Several studies showed the effects of glucocorticoid hormones on the hippocampus. It has been reported that the chronic administration of high dose glucocorticoids (GC) results in the degeneration of pyramidal neurons. However, bilateral adrenalectomy has been shown to damage the hippocampal neurons. Although the effects of long-term adrenalectomy have been studied extensively, there are few publications on the effects of short-term bilateral adrenalectomy (ADX). We aimed to investigate the effects of ADX on levels of pro-inflammatory cytokines interleukin-1β (IL-1β), interkeukin-6 (IL-6) and tumor necrosis factor-α (TNF-α); levels of growth factors, response of microglia and astrocytes to neuronal death, and oxidative stress markers; reduced glutathione (GSH), catalase (CAT) and malondialdehyde (MDA) over the course of time (0.5, 2, 4, 12 hours, 1, 3, 7, 14 days) in hippocampus of male Wistar rats.

Results showed significant elevation of pro-inflammatory cytokines IL-1β at 4 hours, 1, 3, 7 days, whereas, IL-6 was significantly increased at 2 and 4 hours, 1 and 3 days in ADX compared to sham rats. After 7 days, elevation of both cytokines returned to control levels. However, TNF-α levels were significantly elevated at 2, 4 and 12 hours and 14 days in adrenalectomized compared to sham rats.

A significant decrease of insulin-like growth factor-1 (IGF-1) levels was observed at 12 hours after ADX and remained consistent at 1, 3, 7, and 14 days compared to sham rats. However, β-nerve growth factor (β–NGF) was significantly reduced in ADX compared to sham rats only at 14 days.

A time dependent increase in degenerated neurons in the dorsal blade of the dentate gyrus (DG) was observed from 3 to 14 days after ADX. An early up-regulation of microglia was observed on day three, whereas, increase in astroglia in the hippocampus was observed at 7 days ADX. After 14 days of ADX we observed a progression of microglia and astroglia up-regulation all over the DG of adrenalectomized rats' hippocampi compared to sham rats.

The antioxidant CAT increased at 3, 7 and 14 days in an effort to protect the neurons. However, oxidative stress was manifested in the ADX compared to the sham rats due to significant decrease of GSH after 7 and 14 days, with a simultaneous increase of MDA, after 7 and 14 days of ADX.

Taking these findings together, we suggest that the early inflammatory components and loss of growth factors might contribute to the initiation of the biological cascade responsible for subsequent hippocampal neuronal cell death in the current neurodegenerative animal model.

Keywords: Glucocorticoids, adrenalectomy, oxidative stress, growth factors, pro-inflammatory cytokines, microglia, astrocytes, neurodegeneration, hippocampus.

Title and Abstract (in Arabic)

دراسة تأثير الاستئصال الثنائي للغدد الكظرية على المدى القصير : التغيرات البيوكيميائية والشكلية في منطقة الحصين في الجرذان

الملخص

تناولت العديد من الدراسات تأثير هرمونات الجلايكورتيكود على منطقة الحصين في المخ ، وتبين أن التعرض لجرعات عالية من هرمونات الجلايكورتيكود لمدة زمنية طويلة يؤدي إلى موت الخلايا العصبية الهرمية . كما تبين أن الاستئصال الثنائي للغدة الكظرية يضر الخلايا العصبية في الحصين . بالرغم من أن آثار استئصال الغدة الكظرية على المدى الطويل قد تمت دراستها على نطاق واسع ، إلا أن عددا قليلا من الدراسات تناولت آثار استئصال الغدة الكظرية على المدى القصير . بالإضافة إلى ذالك ، إن الهدف من هذه الدراسة هو البحث عن آثار الاستئصال الثنائي للغدة الكظرية على المدى القصير على مستويات السيتوكينات المحفزة للالتهاب مثل سيتوكينات (IL-1β و IL-6 و TNF-α) ، ومستويات عوامل النمو . كما تركز الدراسة على استجابة الخلايا غير العصبية (Microglia & Astrocytes) لموت الخلايا العصبية ، و تأثير عملية الإجهاد التأكسدي مثل الجلوتاثيون (GSH) ونشاط إنزيم الكاتاليز (CAT) والملونديالديهايد (MDA) . وتتابع الدراسة هذه التأثيرات في منطقة الحصين من مخ ذكور الجرذان من نوع ويستار (Wistar) على فترات زمنية مختلفة وهي (0.5 و 2 و 4 و 12 ساعة و 1 و 3 و 7 و 14 يوما) بعد الاستئصال الثنائي للغدة الكظرية .

وقد أظهرت نتائج الدراسة ارتفاعا كبيرا في مستويات السيتوكينات المحفزة للالتهاب ؛ حيث لوحظ ارتفاع مستوى IL-1β بعد 4 ساعات وخلال بعد 1 و 3 و 7 أيام من الاستئصال الثنائي ، في حين أن مستوى IL-6 تزايد بشكل ملحوظ بعد 2 و 4 ساعات وخلال 1 و 3 أيام

في الجرذان التي استئصلت منها الغدد الكظرية (ADX) مقارنة بالجرذان التي أجريت لها العملية دون أن يتم الاستئصال (Sham) . وبعد 7 أيام لوحظ أن هذا الارتفاع في مستويات-IL 1βوIL-6 يعود إلى مستويات مجموعات (Sham) من الجرذان . أما مستويات سيتوكين TNF-α فقد ارتفعت بشكل كبير بعد 2 و 4 و 12 ساعة وكذلك بعد 14 يوما من الجراحة المطبقة على جرذان مجموعات (ADX) مقارنة بجرذان مجموعات (Sham) .

وقد لوحظ انخفاض كبير في مستويات بروتين IGF-1 ابتداء من 12 ساعة بعد الاستئصال (ADX) واستمر ثابتا بعد 1 و 3 و 7 و 14 يوما من الجراحة مقارنة بجرذان مجموعات (Sham) . أما مستويات بروتين (βNGF) فقد انخفضت بشكل ملحوظ في مجموعات (ADX) مقارنة بمجموعات (Sham) بعد 14 يوما فقط من الجراحة.

وقد أظهرت الدراسة ارتفاع تدريجي في موت الخلايا العصبية في الشفرة الظهرية للتلفيف المسنن (dentate gyrus) من الحصين بعد 3 أيام ولغاية 14 يوما من استئصال الغدة الكظرية . وقد لوحظ زيادة مبكرة في نشاط الخلايا الدبقية الصغيرة (Microglia) في اليوم الثالث بعد الجراحة ، في حين لوحظ ازدياد نشاط الخلايا النجمية (Astrocytes) في الحصين بعد 7 أيام من الاستئصال (ADX) . أما بعد 14 يوما من الجراحة فقد ازداد نشاط الخلايا غير العصبية الدبقية الصغيرة (Microglia) والنجمية (Astrocytes) ليشمل جميع أنحاء التلفيف المسنن (dentate gyrus) من الحصين في مجموعات (ADX) مقارنة بمجموعات (Sham).

وقد زاد نشاط إنزيم الكاتاليز (CAT) المضاد للأكسدة بعد 3 و 7 و 14 يوما من الجراحة في محاولة لحماية الخلايا العصبية من الإجهاد التأكسدي الذي ظهر بوضوح في مجموعات (ADX) مقارنة بمجموعات (Sham) بسبب الانخفاض الكبير في مستويات

الجلوتاثيون (GSH) ، وكذلك ارتفاع مستويات الملونديالديهايد (MDA) الدالة على اختلال الغشاء البلازمي للخلايا بعد 7 و 14 يوما من الجراحة .

بناءً على ما سبق ، فقد أظهرت الدراسة أن الاستئصال الثنائي للغدة الكظرية على المدى القصير يؤدي إلى ارتفاع مبكر في مستويات السيتوكينات المحفزة للالتهاب ، وانخفاض في عوامل النمو متبوعا بموت الخلايا العصبية وزيادة نشاط الخلايا غير العصبية إضافة إلى زيادة الإجهاد التأكسدي في منطقة الحصين . وبناء على هذه النتائج يمكن أن نستنبط أن حدوث الالتهاب في وقت مبكر وفقدان عوامل النمو يسهم في بدء سلسلة من العمليات البيولوجية المسؤولة عن موت الخلايا العصبية في الحصين في هذا النموذج الحيواني التجريبي لموت الخلايا العصبية . ومن ثم يمكن التكهن بأنه في العديد من أمراض موت الخلايا العصبية مثل مرض الزهايمر؛ قد تؤدي الزيادة المزمنة في هرمونات الغدة الكظرية - مع / أو انخفاض مستقبلاتها - إلى نشوء آليات التهابية وفقدان عوامل النمو مثل IGF-1 مما ينتج عنه موت الخلايا العصبية في الحصين.

مفاهيم البحث الرئيسية: جلايكورتيكود ، استئصال الغدة الكظرية ، الإجهاد التأكسدي ، عوامل النمو ، السيتوكينات المحفزة للالتهاب ، الخلايا الدبقية الصغيرة ، الخلايا النجمية ، موت الخلايا العصبية ، الحصين .

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Dedication

To my beloved parents and husband.

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
AD	Alzheimer's Disease
ADX	Short-term Bilateral Adrenalectomy
AMPA	Amino-Hydroxy-Methyl-Isoxazolepropionic Acid
BSA	Bovine Serum Albumin
CA	Cornu Ammonis
CAT	Catalase
CORT	Corticosterone
CRH	Corticotropin-Releasing Hormone
CNS	Central Nervous System
CX3CL1	Fractalkine
DA	Dopamine
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
EC	Entorhinal Cortex

ELISA	Enzyme-linked Immunosorbent Assay
GC	Glucocorticoids
GFAP	Glial Fibrillary Acidic Protein
GSH	Reduced Form of Glutathione
GSSG	Glutathione Disulfide
H_2O_2	Hydrogen Peroxide
HPA	Hypothalamic–Pituitary–Adrenal Axis
HRP	Horseradish Peroxidase
H_2SO_4	Sulfuric Acid
Iba1	Ionized Calcium-Binding Adapter 1
IGF-1	Insulin-like Growth Factors 1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	inducible Nitric Oxide Synthase
LEC	Lateral Entorhinal Cortex
LTP	Long Term Potentiation
MAPK	Mitogen-Activated Protein Kinase

MDA	Malondialdehyde
MEC	Medial Entorhinal Cortex
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NMDA	N-methyl-d-Aspartate
β-NGF	Beta Nerve Growth Factor
NeuN	Neuronal Nuclei Antibody
$\bullet O_2$-	Superoxide Radical
$^1O_2$	Singlet Oxygen
•OH	Hydroxyl Radical
PBS	Phosphate Buffered Saline
PD	Parkinson’s Disease
ROS	Reactive Oxygen Species
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TNF-α	Tumor Necrosis Factor -Alpha
UAEU	United Arab Emirates University

Chapter 1: Introduction

1.1 Historical Evolution of the Hippocampus

1.1.1 Origin of Name

History recalls the earliest description of the hippocampus located in the temporal horn of the lateral ventricles, by anatomist Julius Caesar Arantius in 1587 in his book "De Humano Foetu" as a seahorse. The origination of the word came from Greek *hippos* "horse" and *kampos* "sea monster" (Bir et al., 2015). However, Arantius lacked the foresight to illustrate his observation. Future scholars were consequently confused as to how this anatomical structure could be perceived (Lewis, 1923).

Figure 1: Human hippocampus compared with a seahorse

Less than two centuries thereafter, in 1729, anatomist Duvernoy was the first to draw the structure of the hippocampus (P. Andersen et al., 2006; Duvernoy et al., 2013). In 1732, Jacques Benigne Winslow proposed the term "ram's horn". Later in 1742, the surgeon de Garengeot used a more mythical term "*cornu Ammonis*" (CA)–

horn of an ancient Egyptian god Ammon. The meaning behind the name was "the hidden"; the god was presented as a ram headed man or sphinx (Olry & Haines, 1998; Pearce, 2001; El-Falougy & Benuska, 2006).

1.1.2 Architecture of the Hippocampus

Pierre Tarin in 1750 differentiated in the hippocampus what he called the "*dentate fascia*" or the DG (El-Falougy & Benuska, 2006). When Felix Vicq d'Azyr identified the hippocampus major and minor in the 18th century, it sparked a hippocampus debate, also known as the Huxley-Owen debate (Bentivoglio & Swanson, 2001). In the 19th century, the two anatomists argued if either of their anatomic findings agreed or opposed Darwin's theory of evolution; whether the human brain was unique compared to the brain of apes, in terms of containing a hippocampus minor "*calcar avis*". It was later settled in favor of Huxley and Darwin (Owen et al., 2009).

Figure 2: Golgi stain of rat hippocampus

In 1873, Golgi discovered a method to understand the nervous system (Swanson, 1999). He introduced silver nitrate impregnation – black reaction, via

microscopic explorations that was modified by Ramon y Cajal and his student, Rafael Lorente de No, to study the cellular architecture of the hippocampus (Ramón y Cajal, 1909). They described the cells and their components in the hippocampus and the two divisions of this structure based on the recurrent circuitry in the pyramidal cell layer. They further defined the properties of small CA1 and large CA3 pyramidal neurons. It was Lorente de No that coined the terminology for the sections; CA1-CA4 (Lorente De Nó, 1934; P. Andersen et al., 2006; Bartsch, 2012).

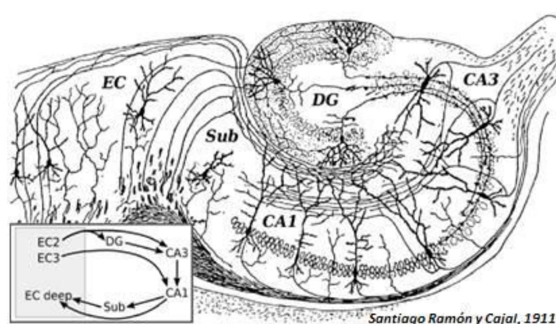

Figure 3: Structure of rat hippocampus

James Papez, in 1937, reported a center of emotion in the hippocampus that was articulated in a circuit. The Papez circuit constitutes of a group of neural projections involved in memory and emotional expression that originates in the cortex and subsequently communicates with the hippocampal formation in the subiculum, in which the mammillary bodies receive a dense input via the fornix. The mammillary bodies in turn project via the mammillo-thalmic tract to the anterior thalamic nucleus, and finally to the cingulum which eventually leads back to the hippocampal formation (Vann & Nelson, 2015; Li et al., 2016). However, MacLean proposed the Papez circuit as part of the limbic system by including the amygdala and septum (MacLean, 1952). Ultimately, in 1998, the Federative Committee on

Anatomical Terminology settled to designate to the term hippocampus as "Hippocampus proper" and/or "Ammon's horn".

1.2 Anatomy

The cerebral cortex is part of the grey matter and composed of un-myelinated nerve cells. It is responsible for higher brain functions, such as; consciousness, language, information processing, sensation, and memory. The outer region of the cerebral cortex is the neocortex folded in cortical structures; the frontal, parietal, occipital, and temporal lobes (Kande et al., 2000; Mai et al., 2015).

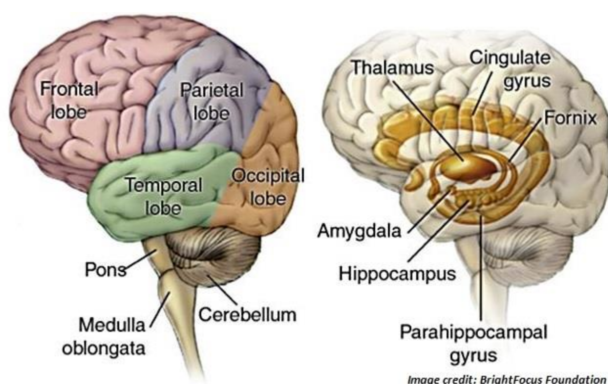

Figure 4: Human brain anatomy

In the fully developed human brain, the hippocampi extend the length of the floor of the inferior horn of the lateral ventricles, which traverse the temporal lobe. The hippocampus is structurally and functionally distinct and has 3 layers; dendrite, pyramidal cell, and axon, that connect to the 6 layers of the neocortex by receiving main afferences from the entorhinal cortex (EC) and sending efferences to other areas through the subiculum (Waxman, 2013).

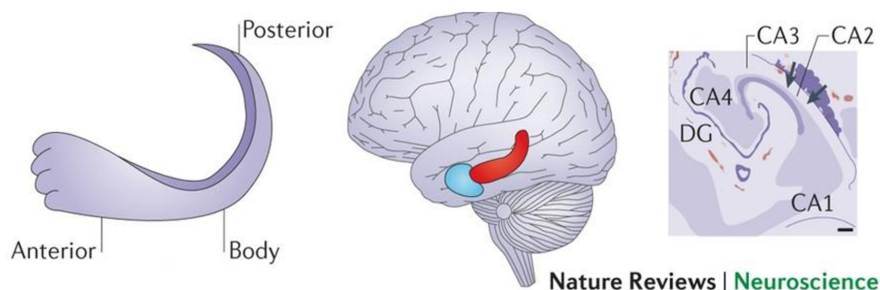

Figure 5: Human hippocampus

The EC is divided into medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC) areas. It has 6 layers; neurons in layers 2 and 3 send projections to the hippocampus, DG, and subiculum, while neurons in layers 5 and 6 receive feedback from CA1 and subiculum (Scharfman, 2011). Much of the hippocampal formation lies in the floor of the temporal horn of the lateral ventricles. It is composed of the DG, CA subfields 1-4, and subiculum.

1.2.1 Hippocampal Layers

The DG has 3 layers: polymorphic layer, *stratum moleculare* contains proximal dendrites of the granule cells, and *stratum granulosum* contains the cell bodies of granule cells and is the principal layer.

The CA has 7 layers: *stratum moleculare, stratum lacunosum, stratum radiatum, stratum lucidum, stratum pyramidale, stratum oriens*, and *stratum alveus.* The *stratum pyramidale* is the principal layer containing the cell bodies of the pyramidal cells. It is divided into four subfields: CA1, CA2, CA3, and CA4. The CA1 region neighbors the subiculum – the inferior part of the hippocampus. The CA3 region is adjacent to the choroid plexus and the fornix. The CA2 region is a boundary between CA1 and CA3, and CA4 is sited in the *hilus* of the DG – the

inside portion of the DG. The hippocampal fissure is a cell-free natural division that separates the DG from the CA1 region (Taupin, 2008).

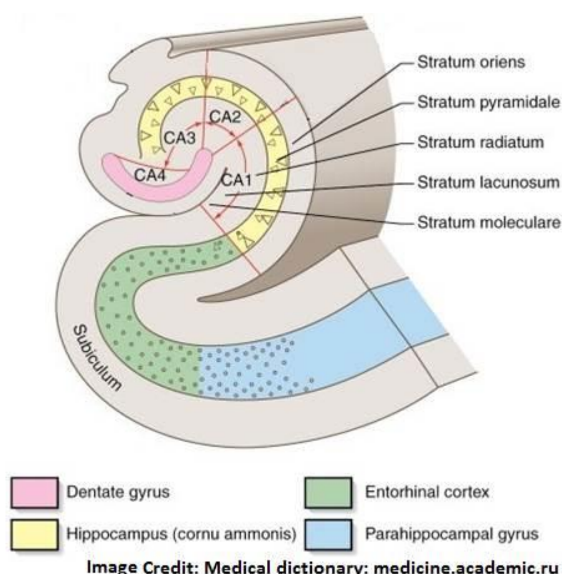

Figure 6: Layers of human hippocampus

1.2.2 The Circuit

The major hippocampal pathways are organized in a trisynaptic circuit; a transverse strip along the septotemporal axis of the hippocampus, with information flowing in a unidirectional way. The trisynaptic circuit was first described by Ramon y Cajal using the Golgi stain method (P. Andersen, 1975). Later, using anatomical and physiological evidence, Per Andersen proposed that thin slices (lamella) could be cut out of the hippocampus perpendicular to its septotemporal axis while preserving all of the synaptic connections. This led to the lamellar hypothesis in which one could assume the hippocampus to be a series of parallel strips operating in an independent manner (P. Andersen et al., 1969; Amaral & Witter, 1989; P. Andersen et al., 2000).

The circuit begins with the first synapse; axons of pyramidal cells of the layer 2 of EC project to the granule cells of the DG by the perforant pathway. The second synapse occurs when axons of granule cells in the DG project to the dendrites of the pyramidal cells of CA3 by the mossy fibers pathway. The third and final synapse allows pyramidal cells of CA3 to project to pyramidal cells of CA1 through collaterals of Schaeffer. The CA1 completes the circuit by feedback to layer 5 of the EC (Eichenbaum, 2004; Taupin, 2008).

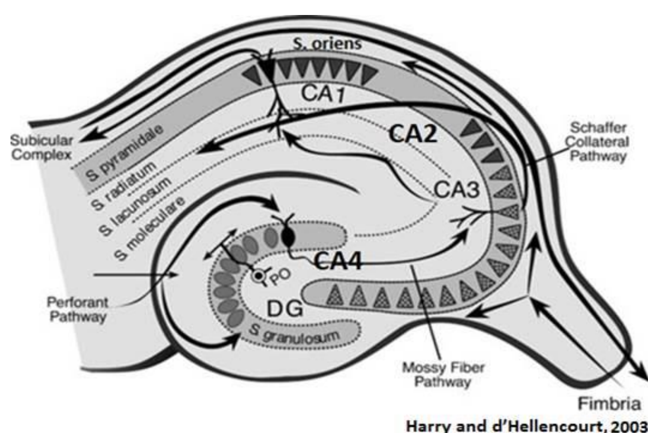

Figure 7: Trisynaptic circuit

However, subsequent neuroanatomical investigations have revealed that hippocampal projections are much more divergent than would be consistent with a strict interpretation of the lamellar hypothesis (Sloviter & Lømo, 2012).There are other intrinsic connections of the hippocampal formation such as: neurons in layer 2 of EC project to the CA3 field via the perforant pathway; neurons from layer 3 of the EC project to CA1 and subiculum via perforant and alvear pathways; CA3 pyramidal cells project heavily onto themselves through recurrent collaterals; CA3 neurons project back to the DG via the hilar mossy cells as feedback; and pyramidal cells in CA1 project to subiculum, then back to EC (Witter, 1993; P. Andersen et al., 2006).

The hippocampal trisynaptic circuit has a well-established role in information processing. It is the main route through which activity flows; information enters the EC from a specific cortical area and can cross the hippocampal circuit via the excitatory pathways and eventually be returned to the cortical area from which it instigated (Nicoll & Schmitz, 2005; Nakashiba et al., 2008; Neves et al., 2008; Daumas et al., 2009). This transformation is presumably essential for enabling the information to be stored as long-term memories.

1.3 Physiology

Studies have shown varied views and perspectives emphasizing prominent features of the hippocampus (Marr, 1971; O'Keefe & Nadel, 1978; Sapolsky, 1996; McNaughton et al., 1996; Leutgeb et al., 2007). It was thought that the function of the hippocampus was to some extent limited to the regulation of olfaction. A characteristic feature of odor memory is to recall emotional experiences; with direct connections between the olfactory bulb and the amygdala and hippocampus (Mouly & Sullivan, 2010). Yet, with the discoveries of Paul Broca and James Papez in identifying the hippocampal formation as part of the limbic lobe and representing the Papez circuit, respectively (Purves et al., 2001), it was broadly and supportably explored as a regulator of emotional behavior.

1.3.1 Declarative Memory

The hippocampus serves a critical role in declarative (explicit) memory – ability to recall everyday facts and events (Squire, 1992). A case study of patient H.M. provided critical information about the hippocampus. H.M. was an epileptic

patient with severe seizures. They began when he was 10 years old, and by the age of 27 his attacks prevented him from living a normal life. Scoville executed an experimental surgery, the bilateral resection of the medial temporal lobes, thus assuaging his epileptic seizures. However, H.M. became amnesic for the rest of his life. He could no longer store new memories. Yet, those before the operation remained intact. He also could not transfer new explicit memory into long term memory. Although, his ability to form new long term implicit memories remained unharmed (Scoville & Milner, 1957; Corkin, 1984). It was concluded that H.M. had severely impaired declarative memory, but his perceptual and cognitive abilities were intact, as were his capacities for working memory and perceptual and motor skill learning (Milner et al., 1968; Eichenbaum, 2004). Yet, it was Brenda Milner and Wilder Penfield that first presented in 1955 at the Transactions of the American Neurological Association with information of the effects of the hippocampal damage on memory in humans (Milner & Penfield, 1955).

In the early 1950's, Penfield began to treat patients for localized injuries that were causing seizures by removing parts of the temporal lobe. However, Milner and Penfield found impairments similar to H.M. – severe, persistent, and generalized damage to recent memory without intellectual loss – in two patients after left temporal lobectomy. To interpret the unexpected memory loss, they hypothesized pre-existing lesions to the hippocampal region of the right hemisphere, that were later confirmed with one of the patients' death (Penfield & Mathieson, 1974; Milner et al. , 1998). It was in 1955 that Scoville asked Milner to examine his patient that started five decades of experimental studies on H.M. (Donkelaar, 2011).

1.3.2 Spatial Memory and Navigation

Besides its role in memory, the hippocampus plays an important part in spatial memory and navigation. Edward Tolman in 1948 presented the concept of a cognitive map by observing rats performing maze tasks. He held the notion that rats learn to navigate mazes by establishing a field map of the environment to reach the prize by means of the shortest route from any certain location (Tolman, 1948; Best & White, 1999). It was not until 1971, O'Keefe and Dostrovsky together discovered the place cells by recording the pyramidal neurons in the CA1 region of the hippocampus of freely moving rats in a bounded area (O'Keefe & Dostrovsky, 1971). O’Keefe and Nadel in 1978 finally established that the hippocampus might act as a cognitive map; a neural representation of the layout of the environment, and they were able to locate these cells (O'Keefe & Nadel, 1978; Burgess et al., 2002). They inserted microelectrodes into different regions of the hippocampus of the rat to record activity. For example; placing a rat in a maze will record the location of activity in the brain that corresponded to where the animal is positioned. These hippocampal neurons became known as “place cells” located in the CA1 region (Corballis, 2014). They fire specifically in patterns known as place fields, corresponding to regions of sensory information in the environment (Leutgeb et al., 2005). When a rat is placed in a fixed environment, spatial firing of place cells becomes intense and rapid. This occurs as the rat’s head is inside a cell-specific region or a firing field (Muller & Kubie, 1987).

Place fields have the ability to change their firing patterns and rates by responding collectively and consistently to large sensory inputs while resisting small changes to the external environment; this is known as remapping – critical for pattern

separation, as it involves transforming an overlapping input pattern into a non-overlapping output, hence, distinguishing memories from one another (Cressant et al., 2002; Jeffery, 2007). These nerve cells in the brain enable a sense of place and navigation and the ability to store information about the environment as a memory (O'Keefe & Conway, 1978).

What May-Britt Moser and Edvard Moser discovered in the medial EC were “grid cells”. They provide the brain with an internal coordinate system essential for navigation (Hafting et al., 2005). These scientists obtained recordings from a different part of the EC; the medial entorhinal cortex (Fyhn et al., 2004). In the first study, they established the presence of certain cells sharing characteristics similar to place cells. In a later study they experimented using larger encounters for the animals to move in, which led them to the discovery of grid cells.

Both discoveries present a hypothesis of how specialized cells can work together to accomplish higher cognitive functions (Moser et al., 2008). Such as, the replay of the activity of place cells during sleep may possibly be a memory consolidation mechanism, where the memory is finally stored in cortical structures. The course by which this might occur is through rapid synaptic modification, primarily within the hippocampus during a waking experience, whereby information is acquired during active behavior. During sleep, synaptic modification is suppressed and the information encoded within the hippocampus is "played back" or re-expressed in circuits by an increase in the firing rate of hippocampal cells; those that are engaged in place-specific activity. These cells have the ability to retain the structure of distributed representations of the visited locations as part of a consolidation process by which hippocampal information is gradually transferred to

the neocortex (Pavlides & Winson, 1989; Treves & Rolls, 1994; Wilson & McNaughton, 1994).

1.4 Memory Formation

Memory is the precise storage and recollection of information. The connection of the hippocampus to memory has made it one of the most studied structures in the brain. Research has shown that the hippocampus is involved in the establishment of stable declarative memories; our capacity for the recollection of unique personal experiences and is involved in the acquisition of semantic or factual knowledge (Cohen & Squire, 1980). It plays a critical role in short-term memory; necessary for long-term memory patterns to be formed.

The underlying mechanisms in which the hippocampus contributes to declarative memory are diverse with differing cognitive processes. When you experience something, the hippocampus learns the information during this experience and then plays the memory back repetitively to the cerebral cortex to formulate a long-term memory, this is consolidation. After a memory has been encoded, the memory undergoes further consolidation, for example while asleep. Finally, the stored memory is relocated to other areas of the cerebral cortex, and the location of encoding of these memories may be a function of the type of memory; that is they are housed in different areas of the cortex. The hippocampus is not only active in encoding memories but also in the retrieval of them. There is evidence to suggest that the hippocampus supports many numerous functions, including, but not limited to, path integration – the ability of place cells to integrate new information on movement (Kubie & Fenton, 2009), and stress response (Sapolsky, 1996).

1.5 The Stress Response

1.5.1 Glucocorticoids

GC or cortisol are hormones secreted in a circadian rhythm. High levels of cortisol are released once a person awakens and are gradually diminished throughout the day. This characteristic of cortisol can be affected by a number of psychological and situational factors. Stress from work has been shown to enhance morning cortisol release (Steptoe et al., 2003). While in situational factors, it can produce a more pronounced cortisol response on work days compared to weekends (Kunz-Ebrecht et al., 2004). GCs are essential for adapting to acute physical stressors; they divert energy to exercising muscle, enhance cardiovascular tone, suppress digestion, growth, and reproduction, suppress immune responses that might otherwise spiral into autoimmunity, and sharpen cognition.

Glucocorticoid hormones are important regulators of hippocampal function with biphasic effects on memory (Conrad et al., 1999). In 1968, McEwen and colleagues used bilaterally adrenalectomized rats to see which parts of the limbic system would uptake and retain circulating radioactive corticosteroids, without the interference and competition of endogenous corticosteroids. McEwen discovered the ability of corticosteroids to bind to the hippocampus (McEwen et al., 1968). It was found that the hippocampus contains the highest concentration of corticosteroid receptors in the brain and provides a negative feedback mechanism, which modifies the hypothalamic–pituitary–adrenal axis (HPA) response (Sapolsky et al., 1984).

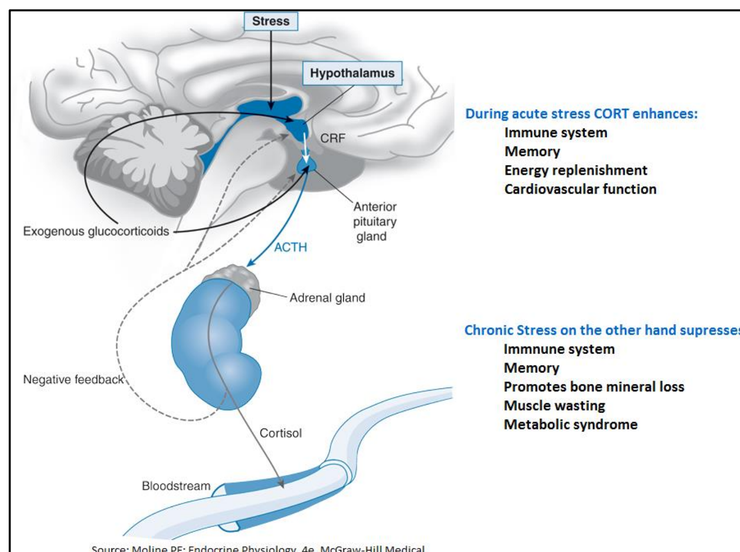

Figure 8: Stress response and HPA axis

During a typical HPA axis stress response, increased availability of circulating GC leads to corticosteroid receptors occupation; this will reduce the release of both corticotropin-releasing hormones (CRH) from the hypothalamus and adrenocorticotropic hormone (ACTH) from the anterior pituitary. The reduction will inhibit the release of corticosteroids from the adrenal cortex, hence, activating a negative feedback loop and decreasing the HPA axis response (McEwen, 2000). This shows how the hippocampus is important to regulate the HPA axis, especially during stress.

Studies have also investigated the release of corticosteroids to awakening in patients with lesions in the temporal lobe (Buchanan et al., 2004; Wolf et al., 2005). They found that damage particularly in the hippocampus does indeed alter the basal cortisol release, which might in turn be affecting the stress response (Buchanan et al, 2009).

1.5.2 Memory and Glucocorticoids

The effects of GC on learning and memory have been examined and experimented on animals, the results supported an inverse-U feature; in the rat, both adrenalectomy and stress levels of GC impair hippocampal-dependent spatial learning (Sapolsky, 1999). Studies in rodents have shown that persistently raised GC have the ability to damage hippocampal neurons (Gould et al., 1990a), while the removal of GCs by bilateral adrenalectomy induces neuronal death (Sloviter et .al, 1993).

Adrenal steroids act on intracellular receptors all through the CNS, therefore, the deletion of GC results in many neurological changes throughout the brain. Throughout growth, GC regulates growth of the cell, its differentiation, and is able to inhibit proliferation of the cells (Meyer, 1985). While in the developed brain, GC diminish the cell death in the hippocampus (Sloviter et al., 1989) and reduce the morphological alterations after adrenalectomy (Gould et al., 1990a).Undeniably, adrenal secreted steroids seem to control memory, learning and consolidation, and influence mood and complex behavioral interactions (McEwen et al., 1986).

1.6 Neurodegeneration

1.6.1 Definition

Neuronal death is recognized in neurodegenerative diseases; however, the mode is unclear. Of those that are well studied include necrosis; a direct result of the

excitotoxic activity of glutamate, and apoptosis; dependent on the activation of apoptotic enzymes – caspases (Gorman, 2008; Czuczwar, 2009).

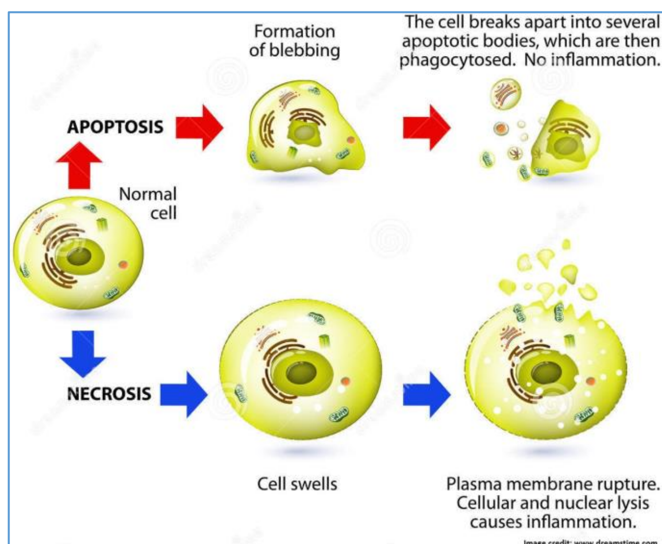

Figure 9: Necrosis vs. Apoptosis

The term 'neurodegeneration' is defined as a chronic and progressive alteration or loss of structure or function of neurons at an amount that causes the neurons to be below their functional threshold (Przedborski et al., 2003; Schloesser et al., 2009).

1.6.2 Neurodegenerative Disorders and Biomarkers

The role of cell death in neurodegenerative diseases remains inexplicable. Most neurodegenerative disorders have an insidious onset while progressively afflicting relentless pain for those suffering and their families. By the time a patient is diagnosed with a neurological illness, widespread neuronal damage has usually already occurred. Accordingly, there is a great need for the discovery of biomarkers that allow early diagnosis and intervention (Gazaryan & Ratan, 2009). Researching

the injured brain has allowed many scientists to study and understand how it works during pathological situations and extrapolate information on its physiology.

Various neurological and neuropsychiatric diseases with extensive damage encompassing the hippocampus led scientists to point out the vulnerability of this region towards pathologies. Magnetic Resonance Imaging (MRI) is a technique used to help with the neuropsychological analysis of hippocampal impairment, and it has helped researchers to derive some cognitive processes that are fundamental for declarative memory (Frisoni et al., 2006). The depictions of neuronal firing patterns in behavior suggest that the hippocampus elicits elemental cognitive processes for declarative memory. Many neuropsychiatric diseases have presented abnormalities in the hippocampus, including Alzheimer's disease and Parkinson's disease (PD) (Theodore et al., 1999; Godsil et al., 2013; Coras et al., 2014).

1.6.3 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder instigated by a progressive and irreversible destructive process of the limbic system and neocortex via amyloid plaque and neurofibrillary tangle formation, loss of synaptic function, mitochondrial damage, activation of microglia and astrocytes, and degeneration of neuronal processes (Schultz et al., 2004; Crews et al., 2010). Neuronal degradation occurs gradually in a process that will lead to the deterioration of cognitive functions. The markers that characterize the development of this process include: amyloid plaques and neurofibrillary tangles (Thal et al., 2002; Serrano-Pozo et al., 2011).

Neurofibrillary tangles are aggregates of hyper phosphorylated and misfolded tau that affect CA1 subfields and layer IV of EC (Braak & Braak, 1991). Hippocampal

circuitry is also compromised; neurofibrillary changes appear in the II layer of EC, plaques begin to form in the perforant pathway, and in a zone that impacts the hippocampal afferents. Thus, the hippocampal formation undergoes severe impairment and is deprived of many afferent and efferent connections leading to an intellectual decline (Van Hoesen & Hyman, 1990; Braak et al., 2006). O'Keefe and co-workers 2008 have showed in a mouse model of Alzheimer's disease that the degradation of place fields correlated with the deterioration of the animals' spatial memory (Cacucci et al., 2008). Yet, there is no immediate translation of such results to clinical research or practice.

It has been proven by many studies the presence of oxidative stress in AD brain such as MDA, a by-product of lipid peroxidation (Pratico & Sung, 2004; Padurariu et al., 2010). The accumulation of antioxidant enzymes such as CAT have also been observed (Omar et al., 1999). The decline of glutathione due to increased oxidative damage localized in the synapses has connected the oxidative stress to AD synaptic loss (Ansari & Scheff, 2010). An index of lipid peroxidation is thiobarbituric acid reactive substance (TBARS) which was found in the hippocampus in significantly high levels (Lovell et al., 1995). This data supports the concept of an Alzheimer's disease hippocampus being under intense oxidative stress.

More evidence lately is linking cytokines such as IL-1, IL-6, and TNF-α released from microglial cells to AD pathogenesis. The initiation of a cascade of neuroinflammation subsequently will contribute to a neuronal damage (Streit et al, 2004; Agostinho et al., 2010). These cytokines such as IL-1, can severely harm memory at high concentrations and are linked to Alzheimer's disease (Williamson et al., 2011). Westwood and colleagues (2014) found that lower levels of IGF-1 are

associated with an increased risk of developing AD, where higher levels of IGF-1 may protect against subclinical and clinical neurodegeneration (Westwood, et al., 2014).

Astrogliosis was observed in human tissue and animal models of AD in the hippocampus (Verkhratsky & Parpura, 2016). An important histological hallmark of the disease is reactive astrocytes that mainly concentrate around senile plaques found in the hippocampus; pathological insults to the central nervous system (CNS) trigger this reaction (Olabarria et al., 2010). These active astrocytes are marked by the increase in production of glial fibrillary acidic protein (GFAP) (Mandybur & Chuirazzi, 1990), elevated cytokines (Mrak et al., 1995), and hypertrophy (da Cunha et al., 1993); this may contribute to the neuroinflammatory response observed in the hippocampus of AD (Lim et al., 2014; Verkhratsky et al., 2014).

Astrocytes display excitability in the form of intracellular calcium concentration increases, which trigger release of glutamate (Shigetomi et al, 2008). The accumulation of calcium in the cytosol is caused by their entry via receptors and channels that are expressed in astrocytes (Verkhratsky et al., 2012). Calcium signaling deregulation is considered to be of fundamental importance for cellular pathology in AD, and the activation of amyloidogenic pathway may function to alter this signaling pathway; hence, being responsible for the learning and memory deficits that occur early during the onset of AD (Green et al., 2007; Berridge, 2010; Grolla et al., 2013). Changes to astrocytes weaken synaptic transmission and affect neurotransmitter homeostasis among other components of AD; thereby responsible for cognitive impairment seen initially in AD (Verkhratsky et al., 2010).

1.6.4 Parkinson's Disease

Parkinson's disease is another neurodegenerative disease that shares many similar characteristics to Alzheimer's disease including the impairment of declarative memory (Carlesimo et al., 2012). In PD, we observe the degeneration of dopamine (DA) neurons in the substantia nigra area of the brain leading to degeneration of the nigrostriatal pathway, or in other words, a loss of nerve terminals and DA which is responsible for the movement disorders, i.e., resting tremor, bradykinesia, and muscle rigidity (Nagatsu et al., 2000a). A key feature of PD is the presence of α-synuclein-positive intracellular inclusions, called Lewy bodies, in DA neurons of substantia nigra, cerebral cortex, and the hippocampus (Sawada et al., 2006). The deposition of these Lewy bodies in the hippocampus is correlated to the severity of cognitive loss in PD patients (Churchyard & Lees, 1997). Non-motor functions such as behavioral disorders and cognitive impairment support the hippocampus in such roles and have become recognized as the foremost cause of disability (Voon et al., 2009; Kehagia et al., 2010). Some of these symptoms occur in early stages of PD and might have a dopaminergic contribution; constipation, rapid-eye movement sleep behavior disorder, depression, hyposmia, and anxiety. They were found to draw a parallel with the progression of PD pathology (Chaudhuri & Schapira, 2009; Hanganu et al., 2014).

Emerging data suggest an interaction between dopaminergic systems and the hippocampus and their role in modulating synaptic plasticity of the hippocampus in memory of PD patients (Calabresi et al., 2013; Foo et al., 2016). The main role of DA in hippocampal synaptic plasticity and memory is long-term potentiation (LTP) in the CA1 region aided by D1/D5 dopamine receptor activation (Hansen &

Manahan-Vaughan, 2014). Another crucial role of DA is the activation of the same receptors on the dendrites of DG neurons accompanied by high-frequency activity of these cells; it will lead to potentiation of the synapses essential for LTP, therefore, affecting spatial memory formation (Hamilton et al., 2010). An interaction between DA and neurotrophic factors occurs in different phases of hippocampal LTP. Many studies show that there is an effect of nerve growth factor (NGF) on DA. One evidence demonstrates that a cortical devascularizing lesion leads to changes in DA levels in the brain, that can be reversed with intraventricular NGF treatment (Maysinger et al., 1992). Further, this effect was also seen in the hippocampus (Paredes et al., 2007). Other studies of neurotrophic factors include *in vitro* studies to determine the effect of IGF-1 on dopaminergic neurons and show support to the survival of these neurons (Knusel & Hefti, 1991). The literature on these growth factors and their involvement in neurodegenerative diseases have been recently expanded and more data is needed to fill in the gap to understand their neuropathology in human diseases (Siegel & Chauhan, 2000). Many reports show evidence of elevated cytokines levels, oxidative stress, activated microglia, and advanced neuronal loss (Lull & Block, 2010). Neurodegeneration occurs due to the activation of microglial cells that elevate the levels of cytokines, such as TNF-α, IL-1β, IL-6 and decrease growth factors such as, NGF (Sriram et al., 2006). Neuroinflammation and oxidative stress may render dopaminergic neurons vulnerable to cell death in PD possibly by triggering the activation of microglial cells (Tieu et al., 2003; Hald & Lotharius, 2005; Miller et al., 2009). Activated microglia observed in the hippocampus of PD patients has shown to have multiple roles; they can phagocytose damaged cells, produce TNF-α, IL-6, IL-1β, superoxide anions, and

neurotrophins. This will promote the neurodegeneration progression (Imamura et al., 2005; Sawada et al., 2006).

The role of astrocytes in PD has not been well understood (McGeer & McGeer, 2008). Research has linked the protein α-synuclein to hippocampus of PD. It is released into the extracellular space and can be taken up by astrocytes which may explain the continuous spread of pathology in PD. The increasing α-synuclein pathology in hippocampal dysfunction was observed in patients with PD and contributes to dementia (Lee et al., 2010; Regensburger et al., 2014; Hall et al., 2014). Studies have suggested an early hippocampal involvement in PD, nonetheless, changes underlying the initial stages of memory and cognitive impairment in PD are yet to be well understood (Bruck et al., 2004; Tam et al., 2005).

1.7 Pro-Inflammatory Cytokines

Stressors have the ability to influence the immune system; both innate and acquired immunity (Chrousos, 2009). Innate immunity works within hours of a stressor and includes granulocytes, natural killer cells, and pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. While acquired immunity takes longer and involves lymphocytes, and other cytokines, such as interleukin-2, interleukin-4, and interleukin-10. The major pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, stimulate the stress system by acting on the hypothalamus to release CRH, on the pituitary to release ACTH, and on the adrenal cortex to release cortisol (Nussdorfer & Mazzocchi, 1998; Fink, 2010). This will eventually lead to the suppression of these cytokines (Chrousos, 1995). However, these cytokines are able to induce a

"sickness behavior"; fever, fatigue, decreased libido, and loss of appetite, during systemic infection and inflammation (Elenkov & Chrousos, 1999; Dantzer, 2001). Cytokines are signaling molecules classified into interleukin, tumor necrosis factor, interferon, and chemokine. Their receptors are located in many parts of the brain including the hippocampus (Gardoni et al., 2011). Thus, cytokines are important for hippocampal functions; learning, memory, and cognitive processes (Vitkovic etal., 2000; Arisi, 2014).

IL-1β is key for synaptic plasticity in the hippocampus to maintain LTP (Schneider et al., 1998). It is produced by glia and neurons and their receptors are localized on the granule cells in the DG. Interleukin-1 may therefore play a crucial role in the CNS (Ban et al., 1991; Ross et al., 2003). Whereas, at pathophysiological levels, IL-1 can cause memory impairment; either by blockade of IL-1 signaling or by impairing Ca^{2+} influx via block of postsynaptic n-methyl-daspartate (NMDA) receptors in the DG (Coogan & O'Connor, 1997; Goshen et al., 2007).

IL-6 is mainly synthesized by astrocytes, but also by microglia and neurons (Oh et al., 2010). Recent studies have explored the role of IL-6 on LTP. By blocking IL-6 after a spatial alteration that is hippocampus dependent, it led to an improvement of long-term memory (Balschun et al., 2004). This study and others have indicated to the negative regulatory role of IL-6 in the CNS, specifically the hippocampus (Campbell et al., 1993; Steffensen et al., 1994). One research showed the ability of IL-6 at high levels to cause neuronal death to developing cerebellar granule neurons (Conroy et al., 2004).

TNF-α is secreted by astrocytes and has a role in preserving synaptic strength by enhancing surface expression of glutamate amino-hydroxy-methyl-

isoxazolepropionic acid (AMPA) receptors (Beattie et al., 2002). TNF-α at pathophysiological levels in hippocampal synapses can inhibit the induction of LTP and this may relate to the inhibition of Ca^{2+} influx or its effect on activation of p38 mitogen-activated protein kinase (MAPK)-dependent mechanism (Cunningham et al., 1996; Barone & Feuerstein, 1999).

Inflammatory Cytokines activate glial cells which in turn have the ability to produce more cytokines; as a result, glial cells undergo gliosis due to TNF-α and IL-1β. Cytokines are not only produced in the CNS, but also peripherally. They are secreted by T-lymphocytes, natural killer cells, and phagocytes that allow these cytokines permeability to the blood brain barrier, hence, their contribution to CNS inflammation (Barone & Feuerstein, 1999).

A study implicated these cytokines to play a role in brain inflammation; by the expression of TNF-α and IL-1α following a surgical trauma to the hippocampus (Tchelingerian et al., 1993).

1.8 Growth Factors

The insulin-like growth factors (IGFs) are polypeptides with a sequence similar to insulin. IGF and IGF receptors are abundantly expressed in the brain where IGF signaling mediates neuronal growth, migration, regeneration, and repair (de la Monte, 2013). IGF is a part of a complex system that has an imperative role in growth and development (Jones & Clemmons, 1995). A number of studies have implicated IGFs in cognitive functions, and proposed their possible effect on cognitive improvement and as compounds that may improve cognitive disorders and even neurodegeneration (Stern et al., 2014).

Nerve growth factor (NGF) is also a polypeptide found in the CNS in which the hippocampus contains one of the highest levels of NGF (Shelton & Reichardt, 1986). NGF is an essential neurotrophic factor for the differentiation and survival of sympathetic neurons (Levi-Montalcini & Angeletti, 1968); it can prompt alterations in certain neurons, therefore, enhancing neuronal plasticity (Sun et al., 1993). The hormonal activities of GCs were investigated if they affect the level of NGF in the hippocampus. It was found that in developing rats, bilateral adrenalectomy considerably diminished NGF levels in the hippocampus (Aloe, 1989). GC and these growth factors play important roles in the brain, nonetheless, their interactions remain unclear and some studies have suggested a relation between them (Sapolsky et al., 1985; Rotwein et al. 1988; Cheung et al., 1994). The consequence of permanent loss of corticosteroids due to bilateral adrenalectomy has shown a neuronal loss in the hippocampus (Adem et al., 1994), yet, information is sparse about these growth factors in the hippocampus following corticosteroid deprivation.

1.9 Glial Cells

1.9.1 Microglia

Microglial cells originate from the bone marrow; they are derived from myeloid progenitors that arise at the time of embryogenesis (Ginhoux et al., 2010). They enter the CNS during early development. In the mature brain they are known as resting microglia, acting through direct cell-cell contact. Upon the appearance of any threat towards the CNS they transform to activated forms important to scavenge for damage and neural protection (Kettenmann et al., 2011; Morriset al., 2013). They

express NMDA receptors in which when stimulated will increase oxidative stress, cytokines, and trigger microglia activation all leading to the induction of cell death of cortical neurons (Kaindl et al., 2012). These receptors and others can be found at neuronal synapses which can regulate microglial responses to inflammation.

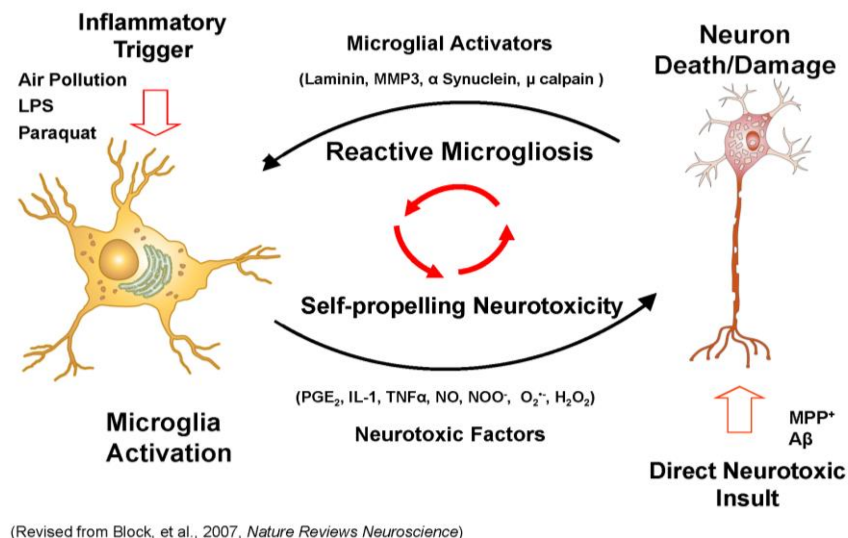

Figure 10: Microglia can become a chronic source of cytokines & ROS

Microglial cells have an important role in memory and learning through their release of cytokines. In one study by Williamson and colleagues in 2011, they were able to directly link the microglia as the only source of IL-1β in response to hippocampus-dependent learning (Williamson et al., 2011).

There has been more evidence that associate different modalities of microglial interactions with synaptic plasticity; in response to a manipulated sensory experience such as light deprivation this caused the microglial highly motile processes to change their morphology and to become less motile. However, light re-exposure reversed these behaviors. This contact could be regulated by learning and memory (Trembla et al., 2010). Other roles include their ability to remove synapses

by phagocytosis (Tremblay & Majewska, 2011; Morris et al., 2013). Another study investigated how microglia and neurons interact and play an important role in cognitive and neuroplasticity processes (Reshef et al., 2014). Fractalkine (CX3CL1) is a chemokine expressed by neurons in the CNS. It participates in inflammatory responses by inhibiting microglia activity in many brain disorders. It binds to its receptor found on microglia in the hippocampus. This signaling pathway has been implicated in synaptic transmission (Paolicelli et al., 2011; Xu et al., 2012). The study also shows that the pathway has a part in seizure-induced microglial activation; providing insight on the role of microglia in neurodegeneration (Ali et al., 2015). An additional neuroprotective role provided by microglia is the production and secretion of growth factors like NGF (Nagatsu et al., 2000b). An activated microglia can also produce reactive oxygen species (ROS) in inflammatory processes leading to oxidative stress (Fischer & Maier, 2015). A cascade of events will ultimately cause neuronal death which is a final pathogenic mechanism in neurodegenerative diseases (Emerit et al., 2004).

1.9.2 Astrocytes

The cell body and the major processes of astrocytes are enriched with GFAP; an intermediate filament protein that is used to distinguish and identify astrocytes in the CNS (Nedergaard et al., 2003).

When afferent pathways are activated in the brain, it results in the release of glutamate, an excitatory neurotransmitter, from synaptic terminals. The released glutamate exerts its effect on target neurons, while the remaining 80% in the synaptic cleft activates the astroglial sodium-dependent glutamate transporters to be removed.

These transporters control the sodium influx, therefore, increase their concentrations in astrocytes. This process eventually is responsible for glutamate turnover to prevent excitotoxicity (Magistretti, 2009). Nonetheless, astrocytes are transporters for many other ions and water. Other functions include the formation of neurovascular units in the brain after an increase in neuronal firing, where rapid vasodilation manifests, thus astrocytes incorporate this neuronal activity with a local blood flow. They also form 50% of synapses and control them. Astrocytes stimulate glycolysis and lactate synthesis; providing active neurons with metabolic substrates. They functions as scavengers of ROS as they are a major source of glutathione (Kimelberg & Nedergaard, 2010; Clarke & Barres, 2013). Astroglia are involved in the pathology of many chronic neurological disorders where they are referred to reactive astrocytes with a characteristic up-regulation of GFAP expression (Oberheim et al., 2008).

1.10 Oxidative Stress

1.10.1 Role in Homeostasis

Redox homeostasis is a complex mechanism that can maintain the balance between ROS production and elimination (Fiedorowicz & Grieb, 2012). ROS are largely generated from mitochondria as by-products of cellular metabolism at physiological levels (Thannickal & Fanburg, 2000), such as; superoxide radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), and singlet oxygen ($^1O_2$). They are scavenged by antioxidants, such as; CAT, GSH, superoxide dismutase, and much more. Yet, a host of environmental neurotoxicants and other various insults may enhance their production in the brain, leading to excessive free-radical release

and resulting in copious amounts of oxidative stress, which can deplete the cell of endogenous anti-oxidative factors (Beit-Yannai te al., 1997; Burton et al., 2010). The capacity of ROS interacting and causing damage to proteins, lipids, and deoxyribonucleic acid (DNA), is a source of significant harmful consequences occurring to the function of these molecules within the cell (Ogino & Wang, 2007).

The CNS is susceptible to oxygen radicals, in particular the brain because of its relatively reduced capacity for cellular regeneration and high metabolic rate. The brain represents only ~2% of the total body weight and demands the highest quantity of oxygen; more than 20% of the total consumption. The two major cell types in the brain, neurons and astrocytes, are responsible for this massive consumption of oxygen and glucose as well. This evidence implicates oxygen radicals in numerous pathological conditions of the CNS (Halliwell, 2006; Shin et al., 2011; Gandhi & Abramov, 2012).

1.10.2 Oxidative Stress in Disease

In PD a reduction in GSH and glutathione disulfide (GSSG) was seen in the substantia nigra (Pearce et al., 1997). These are one of the initial well-known biochemical indicators of degeneration, and the extent of depletion match the level of severity of the disease. This was also observed by Sian and colleagues in PD. They were able to measure GSH levels in the brains of dying patients with PD (Dexter et al., 1989; Sian et al., 1994). They came to a conclusion that GSH in the brain of PD is consistent with the concept of oxidative stress as a major component in the pathogenesis of cell death in PD.

Oxidative stress, manifested by lipid peroxidation among other indices, was observed and suggested to be a key feature in AD brain (Bradley-Whitman & Lovell, 2015; Luca et al., 2015). For example, a marker for lipid peroxidation, MDA, has been identified in the cortex and hippocampus of patients with Alzheimer's Disease (Butterfield et al., 2002). The antioxidant enzyme, such as CAT, displayed reduced activities in affected brain regions in Alzheimer's disease (Pappolla et al., 1992).

The initiation and progression of epilepsy has implicated oxidative stress as an etiologic factor in seizures; where prolonged upsurges in ROS carry an integral risk of increasing neurodegeneration such as that seen in epilepsy (Ashrafi et al., 2007). Oxidative stress in relation to cell death is a contributing factor in epileptogenesis, because neuronal cells are considered vulnerable to oxidative damage and are a consequence of epileptic seizures (Shin et al., 2011). Nevertheless, raised oxidative stress does not demonstrate that it is involved in the neurodegeneration that is associated with these disorders. Cells have developed several defense and repair mechanisms to deal with oxidative stress and associated damage, however in these conditions, antioxidant defense molecules that would normally neutralize the injuries of ROS, are reduced.

1.11 Animal Models of Hippocampal Neurodegeneration

Animal models have been used in an attempt to explain a precise cause and mimic important aspects of pathologies associated with disorders, such as those of the CNS, and have been successful to reproduce key aspects of disorders in neuroscience areas. Using animal models in research allows for experimental manipulation that can produce a hippocampal neuronal loss with possible underlying

mechanisms that may be similar to those portrayed in neurodegenerative diseases. For the past few decades, long-term bilateral adrenalectomy-induced death of hippocampal neuron had been used as a model to study the regulation of apoptosis in adult neurons (Bye & Nichols, 1998; Bye et al., 2001), behavioral learning in spatial memory tasks (Conrad & Roy, 1993), and many more.

Chronic studies on adrenalectomy provide insight into functions of the hippocampus and shed light into mechanisms of neuronal death (Spanswick & Sutherland, 2010). It was discovered that long-term adrenalectomy of normal rats caused hippocampal granule cell loss, suggesting that this phenomenon might be a useful model of experimentally controlled neuronal death relevant to neurodegenerative disorders (Sloviter et al., 1989; Gould et al., 1990a; Sloviter et al., 1993).

Spanswick and colleagues in 2011 used long-term adrenalectomy as a novel animal model to examine whether neuronal replacement in the hippocampus can reverse memory deficits caused by selective degeneration of hippocampal neurons (Spanswick et al., 2011). Another study used long-term bilateral adrenalectomy to increase our understanding of how neurons in the hippocampus die and the protective effect of growth factors against apoptosis (Nichols et al., 2005). The possibility that this cell death involves a biochemical cascade pertinent to neurodegenerative diseases proposes that this model may be valuable for studies of neuronal death and its prevention. Another reason to use ADX is the discrepancy in surgical procedures used to cause damage to neural structures resembling lesions seen in neurodegenerative diseases. Such practice may influence the severity and degree of loss, leading to inconsistencies in results across similar studies. The factors that

might have contributed to the variations in research outcomes could include, efficacy of surgery and difference in sensitivity of techniques to measure extent of neuronal injury (Zola et al., 2000). An example of this is using the anti-mitotic agent colchicine injections in the DG of rats as a technique to remove granule cells and study spatial memory (Xavier et al., 1999), which may lead to variable results from memory experiments due to the unknown extent of damage produced in the hippocampus (Jeltsch et al., 2001). This obstructs the ability to conclude definite conclusions about the differences in study results. Despite these limitations, overall findings support the suggestion that the DG plays an important role in memory (Sloviter et al., 1993).

1.12 Study Rationale and Aim

Long-term bilateral adrenalectomy has been shown to damage the hippocampal neurons (Sloviter et al., 1989; Sapolsky et al., 1991; Adem et al., 1994; Krugers et al., 1994; Sousa et al., 1997; Nichols et al., 2005; Sugama et al., 2013). Although the effects of long-term adrenalectomy have been studied extensively there are few publications on the effects of short-term bilateral adrenalectomy (Jaarsma et al., 1992; Vazquez et al., 1993; Hamadi et al., 2016). We hypothesize that early rise of neuroinflammation and a reduction in neuronal growth factors trigger hippocampal neuronal cell death following ADX. In the present study we aimed to investigate the effects of short-term bilateral adrenalectomy of the hippocampus of male Wistar rats at different time points (0.5, 2, 4, 12 hours, as well as 1, 3, 7, 14 days) on the:

1. Levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α.
2. Levels of growth factors IGF-1 and β-NGF.
3. Response of microglia and astrocytes to neuronal cell death.
4. Oxidative stress markers GSH, CAT, and MDA.

Chapter 2: Methods

2.1 Animals

Male Wistar rats (n = 8-10) were obtained from the United Arab Emirates University Animal House. At the onset of the experiment the rats weighed approximately 160 – 170 grams. Rats were housed five per cage in a 12-hour light/dark cycle and received *ad libitum* access to food and water for the duration of the study. Rats were maintained in a temperature of 22°C. All experimental procedures were conducted in accordance and with the approval of the UAEU Animal Ethics committee guidelines.

2.2 Time-Course of Adrenalectomy

Rats underwent either ADX or corresponding Sham surgery (controls). Rats were weighed and divided in their respective cages 3 days before the surgery to accustom them to human handling and exposure; this helped to minimize any stress they may have experienced and will not interfere with the experiment. Both handling acclimation and housing accommodations were performed to minimize stress, which can activate the HPA axis and GC secretion. Anesthesia was induced using a 6 mg/ml concentration of pentobarbitone (35 mg/kg, intraperitoneal). A surgical plan was not needed to be maintained for the surgical procedure, as the procedure duration lasted 20 minutes and the effect of the anesthesia reached half-life after 30 minutes.

Upon induction, the flanks were shaved and swabbed three times with alcohol (70% ethanol). ADX involved making an approximately 2 cm long incision through

the skin in each flank using a number 11 scalpel. Muscle was then blunt dissected. The adrenal glands were removed using organ forceps. The muscle wall of the peritoneal cavity was sutured closed with Clini-Gut® (19mm, CliniSut Sutures). Skin was closed using AutoClip for wounds (9mm, Clay Adams®), and swabbed with Betadine® (povidone-iodine).

Sham surgery was performed in a similar fashion without the removal of the adrenal glands. Rats were housed in opaque cages under ultraviolet light for warming post-surgery to reduce environmental stress (Conrad & Roy, 1993). Immediately following surgery, all rats were given Trimol® (co-trimoxazole: sulfamethoxazole 40mg/ml + trimethoprim 8mg/ml) 10 ml for 3 days, mixed with their drinking water, prophylactically against post-surgical infection (Spanswick et al., 2007; Spanswick et al., 2011).

All ADX rats were provided 0.9 % saline in lieu of drinking water for the remainder of the experiment in order to maintain electrolyte balance and prevent the deleterious effects of sodium chloride insufficiency (Krause & Sakai, 2007; R. S. Sloviter et al., 1989). Rats were weighed before the surgery and at time of sample collection.

2.3 Sample Collection

Upon euthanasia, at different time points; 0.5, 2, 4, 12 hours, as well as 1, 3, 7, 14 days, the animals were sacrificed by an overdose of pentobarbitone. Blood from the vena cava was collected and placed in clot activator gel tubes (BD Vacutainer®). Blood samples were centrifuged at 15,000 rpm (20879 G) for twenty minutes. The separated serum was removed and stored at -40 °C until the samples

were assayed. The head was placed on ice while removing the brain. Using surgical scissors, we made a midline incision in the skin and flipped the skin over the eyes to free the skull. The skull was removed by making a cut through the anterior part, between the eyes. We then tilt the parietal and frontal bones to break them off while taking care of the meninges. Finally, we used a curved narrow forceps under the olfactory bulb to break the optic nerves. Immediately after placing the brain on a petri dish filled with ice, the cerebellum was dissected. The cortex was opened from the midline and removed from both sides of the brain to free the hippocampus. The hippocampus was dissected by rolling it out of the brain to free it from the cortex (Spijker, 2011).

The hippocampi were stored in 2 ml microtubes then submerged in liquid nitrogen for flash freezing. All samples were stored in -40°C.

2.4 Hippocampal Tissue Homogenate

For the assays, the hippocampi samples weighed approximately 80-100 milligrams. Protein extraction was performed by incubating the tissues in Thermo protein extraction reagent® (T-PER) buffer that contain 25mM bicine and 150mM sodium chloride (pH 7.6), it was added in a ratio of (1:5) weight to microliter volume and with (1:100) microliter volume of protease inhibitor. The incubation with the buffer for 30 minutes on ice was done prior to homogenizing individual samples with ultra-sonication using a sonic ruptor 250 (OMNI®) for 15 seconds at minimum speed. To facilitate cross-linking, samples were incubated for another 30 minutes on ice, and centrifuged in a micro centrifuge for 15,000 rpm (20879 G) (eppendorf® 5417R) for 30 minutes at 4°C. Then the supernatant was collected and placed in 2

aliquots to reduce the amount of freeze – thaw cycles for multiple quantitative analyses.

2.5 Corticosterone Analysis

To assess the effectiveness of ADX, approximately 2.0 ml of blood was collected from each rat. Blood samples were centrifuged at 15,000 rpm for twenty minutes. The separated serum was used to measure the level of circulating corticosterone (CORT). A competitive ELISA Corticosterone using R&D Systems® was performed according to kit instructions.

The quantitative assay was based on a competitive binding technique. A sheep polyclonal antibody specific to CORT bounded to the microplate, which was commercially coated with a donkey anti-sheep IgG antibody. That allowed the CORT present in the samples to compete with horseradish peroxidase – labeled corticosterone for sites on the polyclonal antibody. A substrate solution consisting of 1:1 hydrogen peroxide and chromogen was added to develop color and determine the bound enzyme activity. The absorbance was read at 450 nm, and the corresponding results were indicative of the CORT concentration in the samples.

2.6 Pro-Inflammatory Cytokines Analysis (IL-1β, IL-6, and TNF-α)

Pro-inflammatory cytokines IL-1β, IL-6, and TNF-α are produced in the brain and are elevated in numerous neuropsychiatric diseases. Various studies on the hippocampus implicated in neurodegenerative diseases have shown an early expression of these cytokines at high levels.

The levels of the cytokines were analyzed using ELISA technique following the kit instructions. The kit was provided by R&D Systems®. The ELISA plates used were Nunc maxisorp®. The ELISA plates were initially incubated overnight at room temperature with the captured/primary antibody for each corresponding cytokine assay; this will later allow the binding of the capture antibody with the present cytokine in the hippocampus homogenate sample. Incubating the plates with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 hours at room temperature the next day will block the plates and prevent any false binding with the walls of the wells. A standard curve is needed to allow the formation of an equation from the straight line; this permits the extrapolation of the cytokine concentrations in each sample by performing a serial dilution of known standard protein concentrations; therefore, standards and samples were added to their respective wells. After this is done, the plates are once again incubated overnight at room temperature. The following day, the plates undergo a series of incubation; with the detection antibody for 1 hour that binds to the cytokines in the sample, and then with horseradish peroxidase (HRP) for 20 minutes. Finally tetramethylbenzidine was added to develop the color and a Stop Solution to terminate the reaction. The plates were read at a wavelength of 450nm.

The cytokine concentrations were calculated against the standard curve and the final results for the cytokines were normalized to the total protein concentrations that were attained from the protein assay for each sample.

2.7 Growth Factors Analysis

2.7.1 Insulin-Like Growth Factor-1 (IGF-1)

IGF-1, a trophic factor for neurons, has also been shown to be an important regulator of cell metabolism, differentiation, and survival.

The Quantikine® ELISA from R&D Systems® assay was used for the quantitative determination of rat IGF-1 in tissue homogenate of the hippocampi. A monoclonal antibody specific for rat IGF-I had been pre-coated onto a microplate. Any rat IGF-I present in the sample was bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for rat IGF-I was added to the wells. Following the formation of bound antibody-enzyme reagent, a substrate solution yielded a blue product that turned yellow with the addition of the Stop Solution. The intensity of the color measured at 450 nm was in proportion to the amount of rat IGF-I bound in the initial step.

2.7.2 β-Nerve Growth Factor (β-NGF)

β-NGF is a trophic factor for neurons and is involved in the maintenance of the sympathetic and sensory nervous systems. It exerts a number of different effects on neurons, such as development, survival, and differentiation. Neurons that fail to obtain sufficient NGF die by apoptosis. It has been shown that adrenalectomy in young rats caused a drastic decrease of the NGF level in the hippocampus (Aloe, 1989).

A DuoSet® ELISA from R&D Systems® assay was used for the quantitative measurement of rat β-NGF in hippocampal tissue homogenate. It employed a specific antibody for rat β-NGF, goat anti-rat β-NGF, coated on a 96-well plate. The β-NGF present in a sample was bound to the wells by the immobilized antibody. Later, biotinylated goat anti-rat β-NGF antibody was added followed by HRP-conjugated streptavidin. The addition of the substrate solution (1:1 tetramethylbenzidine and H_2O_2) allowed color development in proportion to the amount of β-NGF bound. Finally, the Stop Solution (2 N sulfuric acid ;H_2SO_4) changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

2.8 Oxidative Stress Analysis

Malondialdehyde is a naturally occurring product of lipid peroxidation. Lipid peroxidation is used as an indicator of oxidative stress in cells and tissues. The measurement of TBARS is a well-established method for screening and monitoring lipid peroxidation.

Cayman's TBARS Assay Kit® was used to assay lipid peroxidation in hippocampal tissue homogenates. An MDA-TBA adduct (Fig. 11) was formed by the reaction of MDA and Thiobarbituric Acid (TBA) when placed in boiling water of 90-100°C and acidic conditions. The adduct was then measured colorimetrically at 540 nm wavelength using the Infinite M200pro (Tecan®).

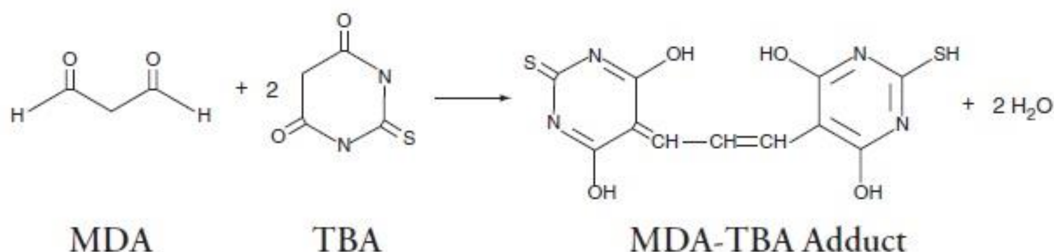

Figure 11: MDA-TBA Reaction

GSH is the key antioxidant in animal tissues. It is present inside cells mainly in the reduced form (90-95% of the total glutathione). Intracellular GSH status appears to be a sensitive indictor of the overall health of a cell, and of its ability to resist toxic challenge.

The glutathione assay kit from Sigma-Aldrich® was used to measure the level of total glutathione (GSSH + GSH) in the hippocampal tissue homogenate. The samples were first deproteinized with 5% 5-Sulfosalicylic Acid Solution, next centrifuged to remove the precipitated protein, and then assayed for glutathione.

GSH was oxidized by the sulfhydryl reagent 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB) and GSSH (Fig. 12). The GSSG formed was recycled to GSH by glutathione reductase in the presence of NADPH (Fig. 13) and the TNB was measured spectrophotometrically. The plate reader was set at a wavelength of 412 nm, with kinetic reading at 1 minute interval for 5 minutes.

Figure 12: GSH Oxidation

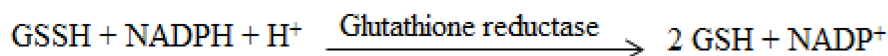

Figure 13: GSSH Reduction

CAT is a ubiquitous antioxidant enzyme that is present in most cells. CAT is involved in the detoxification of H_2O_2, a ROS, which is a toxic product. This enzyme demonstrates catalytic activity and peroxidatic activity (Fig. 14).

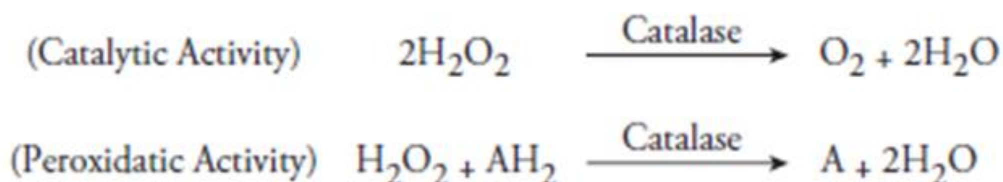

Figure 14: CAT Detoxication of Hydrogen Peroxide

Cayman's Catalase Assay Kit® was employed to measure CAT activity in tissue homogenates of the hippocampus. It utilized the peroxidatic function of CAT for determination of enzyme activity. The method was based on the reaction of CAT with methanol in the presence of H_2O_2. Formaldehyde was produced and measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole (Purpald) as the chromogen. It specifically formed a bicyclic heterocycle with aldehydes, which upon oxidation changed from colorless to a purple color. The absorbance was read at 540 nm wavelength.

2.9 Immunohistochemistry

In order to determine a time-course of ADX-induced cell death, ADX and sham operations were performed on male Wistar rats (n=32) for immunohistochemical examination. At varying time points; 1 day, 3 day, 7 day, and 14 day, animals were anesthetized with a lethal dose of sodium pentobarbital (>35 mg/kg, intraperitoneal). A perfusion-fixation procedure was done transcardially; by first cutting open the right atrium to allow the removal of circulating blood, with 50 ml of 0.1 M phosphate-buffered saline perfused by inserting a needle through the ascending aorta, followed by 300 ml of 4% formaldehyde in 0.1 M PBS to fix the tissue and prevent any denaturing of proteins.

Brains were removed and stored in the same fixative for 1 week. The tissues were then run through an ethanol series for dehydration, cleared in 100% xylene, and embedded in paraffin wax. Blocks were ready to be sectioned on a rotating microtome. Coronal Sections were taken at 1μm and mounted on gelatin subbed slides. The tissues were then prepared for fluorescent labeling.. The sections were deparaffinized with xylene series at 5 minutes each, rehydrated with an ethanol series (100, 90, 80, and 70%) also at 5 minutes each, and washed in distilled water for 5 minutes. Then tissues underwent an antigen retrieval technique with 10mM Sodium Citrate (pH 6), trailed by microwave heating for 1 minute at high power followed by 10 minute at low power; this allowed for proficient immunostaining. Finally, sections were washed with 0.1 M PBS (pH 7.4) and cooled down at room temperature.

Double immunofluorescence labeling was done by first blocking the sections with 1% BSA buffer for 30 minutes at room temperature. Afterward, the primary antibodies were added to the slides and left for incubation overnight at 4°C. Ionized

calcium-binding adaptor molecule-1 (Iba-1) is a protein that is specifically expressed in microglia and is up regulated during the activation of these cells, and these antibodies are specifically reactive to microglia and are also appropriate for immunodouble staining of brain tissues. Neuronal Nuclei (NeuN) antibody specifically recognizes the DNA-binding, neuron-specific protein NeuN, which is present in the brain. GFAP is a gene that encodes one of the major intermediate filament proteins of mature astrocytes. It is used as a marker to distinguish astrocytes from other glial cells during development.

So for the primary antibodies we used rabbit polyclonal anti-ionized calcium-binding adaptor molecule 1 (Iba1, Wako, MA, USA 1:2000) mixed with mouse monoclonal anti-neuronal nuclear antigen (NeuN, Millipore, MA, USA, 1:1000). And rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, Dako, Copenhagen, Denmark, 1:1000) was mixed with mouse monoclonal anti-neuronal nuclear antigen (NeuN, Millipore, MA, USA, 1:1000). The following day, the sections were washed several times in PBS, and then tissue sections were incubated with secondary antibodies. We used donkey anti-rabbit conjugated to Alexa 488 (Invitrogen, Paisley, UK, 1:200) mixed with donkey anti-mouse conjugated to Rhodamine (Jakson, Pennsylvania, USA, 1:100). The antibodies were diluted in PBS-Triton X-100 0.3% for 1 hour at room temperature. Later, the sections were washed in PBS several times; and cover slipped with a fluoromount medium.

2.10 Statistics

All statistical analyses were conducted using the IBM SPSS Statistics program version 21; we employed *t*-test for equality of means with a confidence

interval 95% of difference. Our data was presented as mean ± standard error mean and a p-value ≤ 0.05 was considered as significant.

Chapter 3: Results

3.1 Corticosterone

ADX surgery resulted in significantly lower CORT levels compared to sham operated rats (Fig. 15). At 0.5h the CORT concentration for ADX rats (1.11 ± 0.59ng/ml) was significantly (P<0.001) lower compared to sham operated rats (64.17 ± 8.26ng/ml). After ADX at 2h (0.03 ± 0.01ng/ml), rats also showed a significantly (P<0.001) reduced CORT level compared to sham operated rats (50.34 ± 7.04ng/ml).

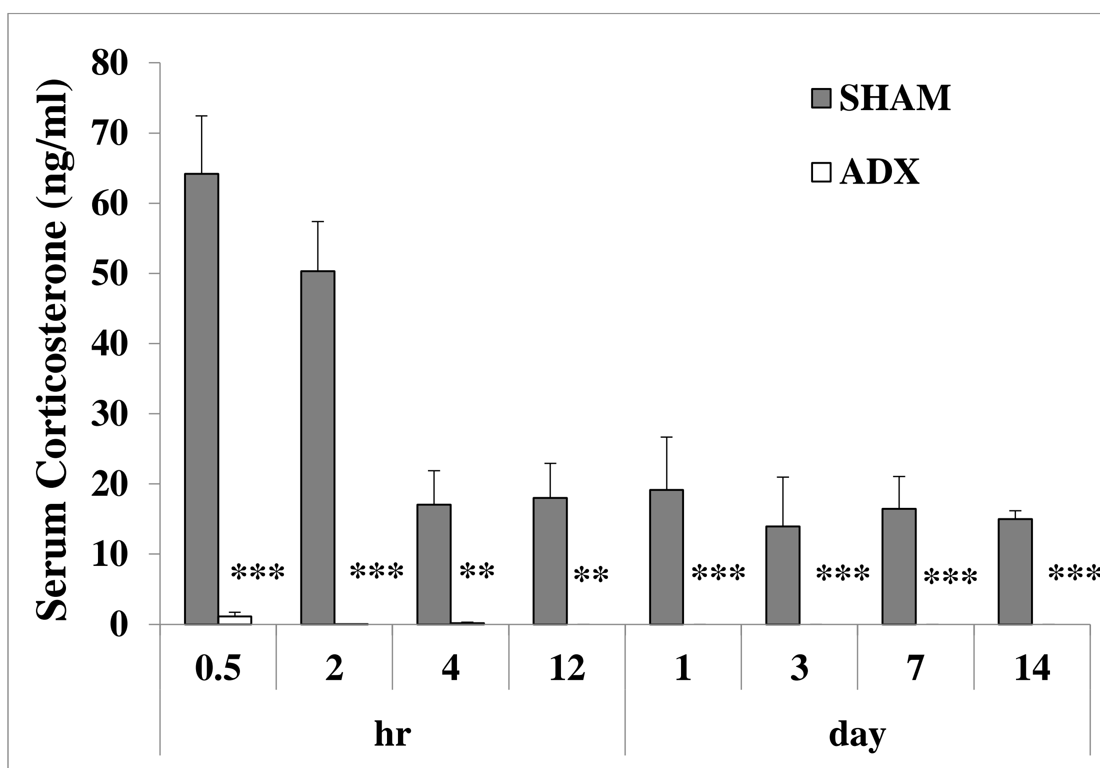

Figure 15: Bar graph showing levels of plasma corticosterone. Levels of corticosterone in the serum of adrenalectomized rats compared to the sham operated rats over the course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days). **P<0.01; ***P<0.001. Data are expressed as mean (±SEM)

The CORT levels were significantly ($P<0.01$) reduced in the serum of 4 hr ADX rats (0.16 ± 0.16ng/ml) compared to sham operated rats (17.07 ± 4.81ng/ml). While at later time-points, circulating CORT levels were undetectable by the kit at concentrations lower than 0.103 ng/ml. A significant effect of post-surgical time was present for sham operated rats. Mean CORT levels were 64.17 ± 8.26, 50.34 ± 7.04, 17.07 ± 4.81, 18.02 ± 4.93, 19.14 ± 7.52, 13.95 ± 7.03, 16.45 ± 4.60, and 15.00 ± 1.18ng/ml at the 0.5-hour, 2-hour, 4-hour, 12-hour, 1-day, 3-day, 7-day, and 14-day time-points respectively.

3.2 Pro-Inflammatory Cytokines

Our results show a clear elevation of pro-inflammatory cytokines at early time points of ADX. IL-6 levels at 2 hours after the surgery were significantly increased ($P<0.05$) in the hippocampus of ADX rats compared to sham operated rats. The increase is seen to be consistent at 4 hours ($P<0.01$), 1 day ($P<0.01$), and 3 days ($P<0.001$). However, 12 hours, 7 days, and 14 days showed no significant changes (Fig. 16).

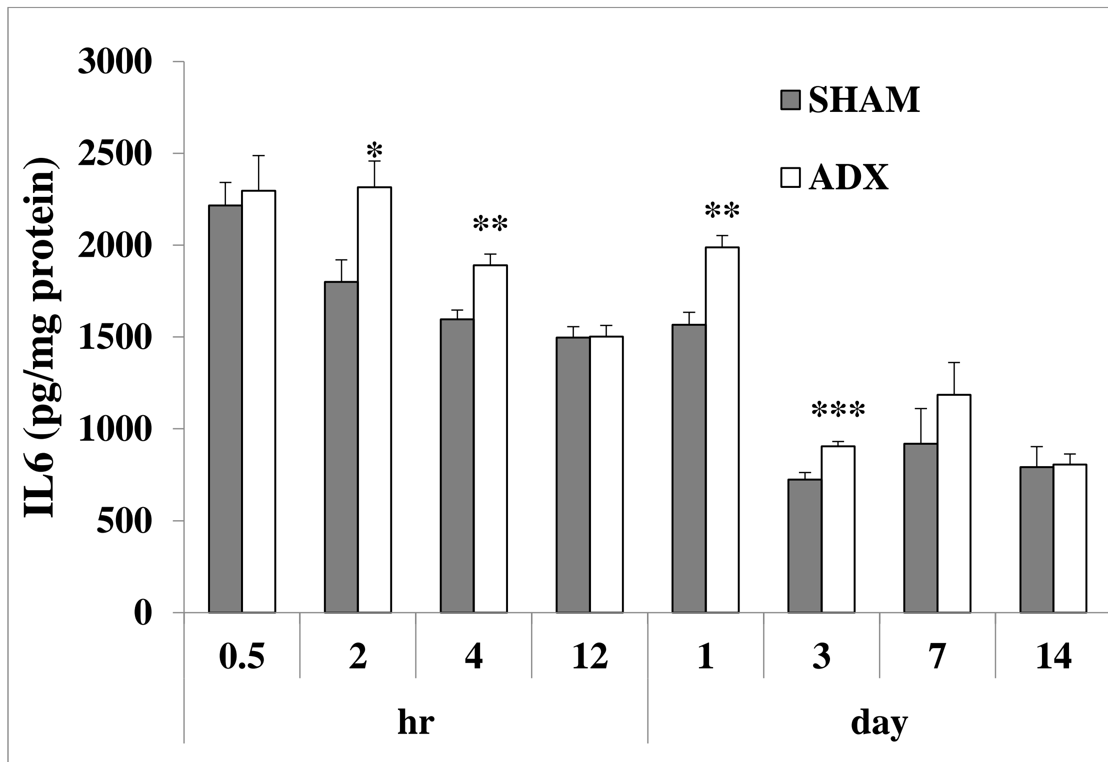

Figure 16: Bar graph showing IL-6 level in the hippocampus of adrenalectomized rats and sham operated rats. IL-6 level was measured by ELISA over the course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days). *P<0.05; **P<0.01; ***P<0.001. Data are expressed as mean (±SEM)

As for the pro-inflammatory cytokine IL-1β, a significant rise in levels were detected in the hippocampus 4 hr (P<0.001), 1 day (P<0.01), 3 days (P<0.05), and 7 days (P<0.001) after ADX compared to the sham animals. No significant changes were observed at the 0.5 hr, 2 hr, 12 hr, and 14 day post ADX (Fig. 17).

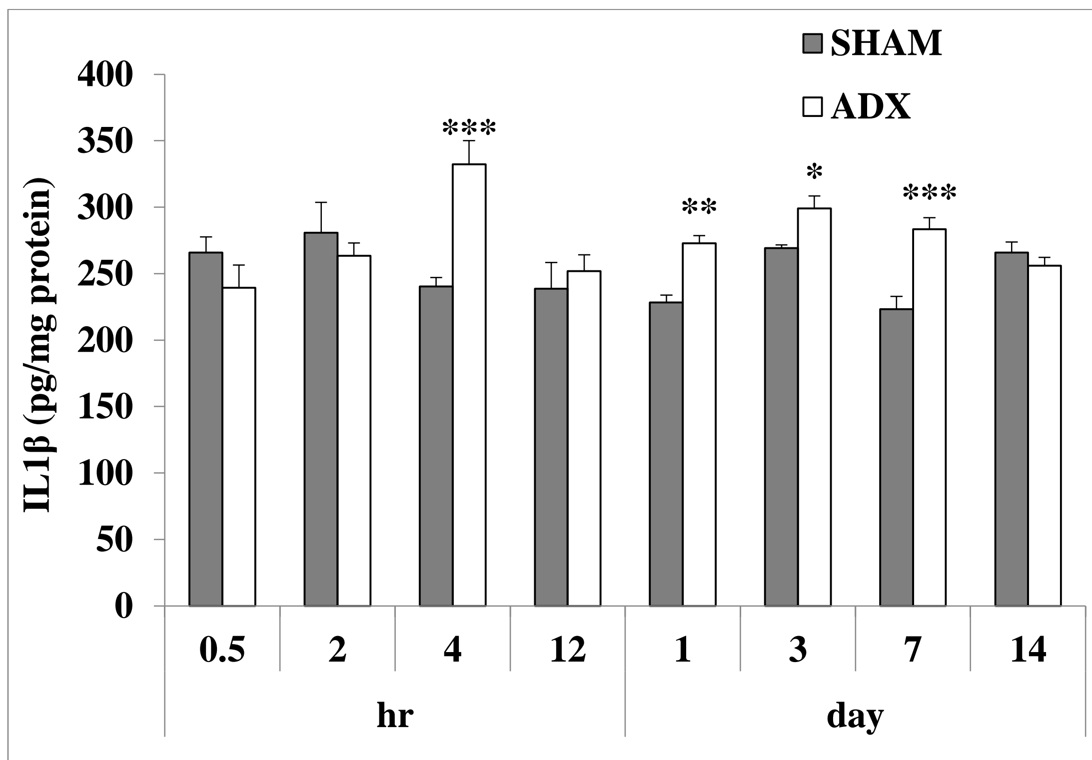

Figure 17: Bar graph showing IL-1β level in the hippocampus of adrenalectomized rats and sham operated rats. IL-1β level was measured by ELISA over the course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days). *P<0.05; **P<0.01; ***P<0.001. Data are expressed as mean (±SEM)

Regarding TNF-α, significant increases were seen as early as 2 hours (P<0.05), 4 hours (P<0.05), and at 12 hours (P<0.001) in the hippocampus of ADX rats. However, except at 14 days no significant differences in ADX compared to sham operated rats were seen at 1, 3, and 7 days(P<0.01), (P<0.05), and (P<0.001) respectively (Fig. 18).

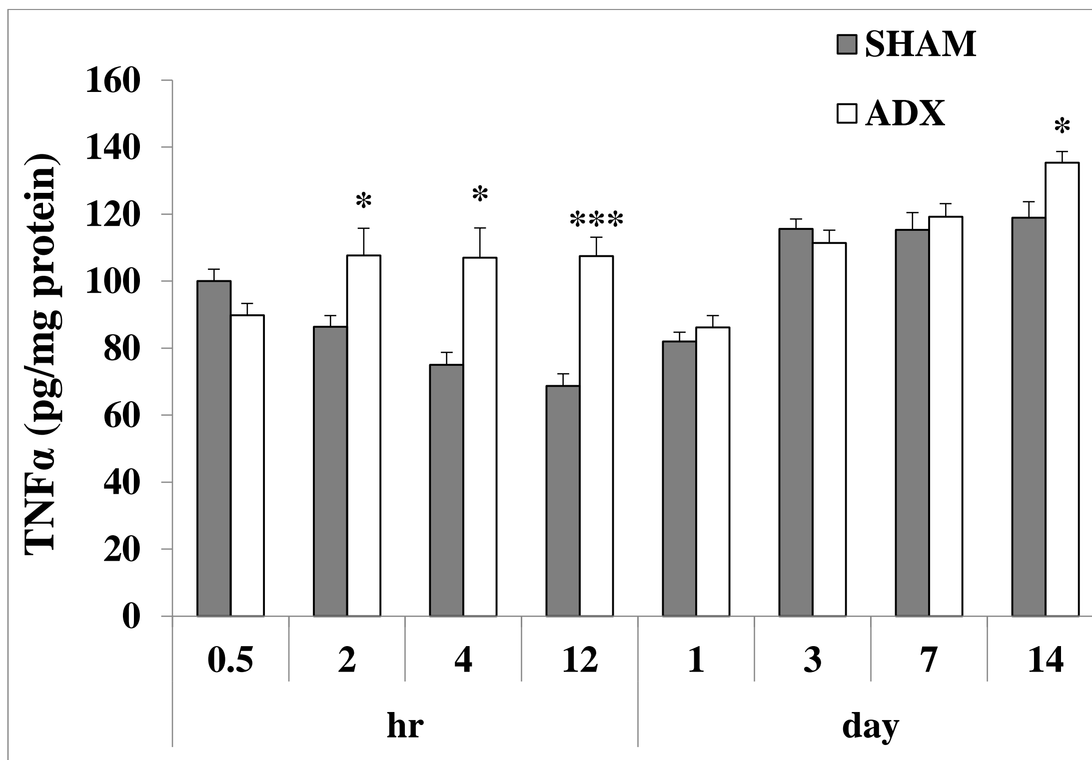

Figure 18: Bar graph showing TNF-α level in the hippocampus of adrenalectomized rats and sham operated rats. TNF-α level was measured by ELISA over the course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days). *P<0.05; ***P<0.001. Data are expressed as mean (±SEM)

3.3 Growth Factors

Using the ELISA method our results showed differences in IGF-1 protein levels. In the hippocampus, the IGF-1 protein levels were significantly decreased compared with those of control animals starting at 12h (P<0.01) after ADX. The results remained consistently low at 1 day (P<0.01), 3 days (P<0.05), 7 days (P<0.01), and 14 days (P<0.05) after ADX compared to control animals (Fig. 19).

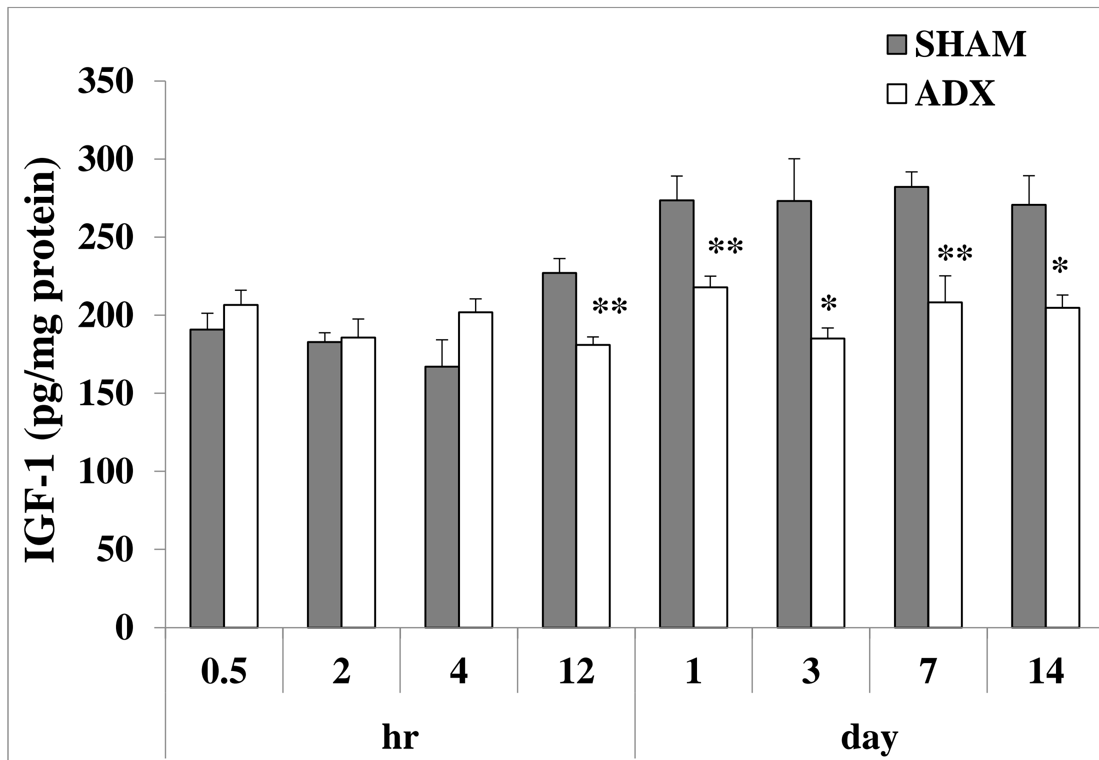

Figure 19: Bar graph showing IGF-1 level in the hippocampus of adrenalectomized rats and sham operated rats. IGF-1 level was measured by ELISA over the course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days). *P<0.05; **P<0.01. Data are expressed as mean (±SEM)

Our results showed a significant decrease (P<0.05) in β-NGF levels only at 14 days after ADX compared to sham operated animals of the same group. There was no statistical difference between the groups prior to 14 days (Fig. 20).

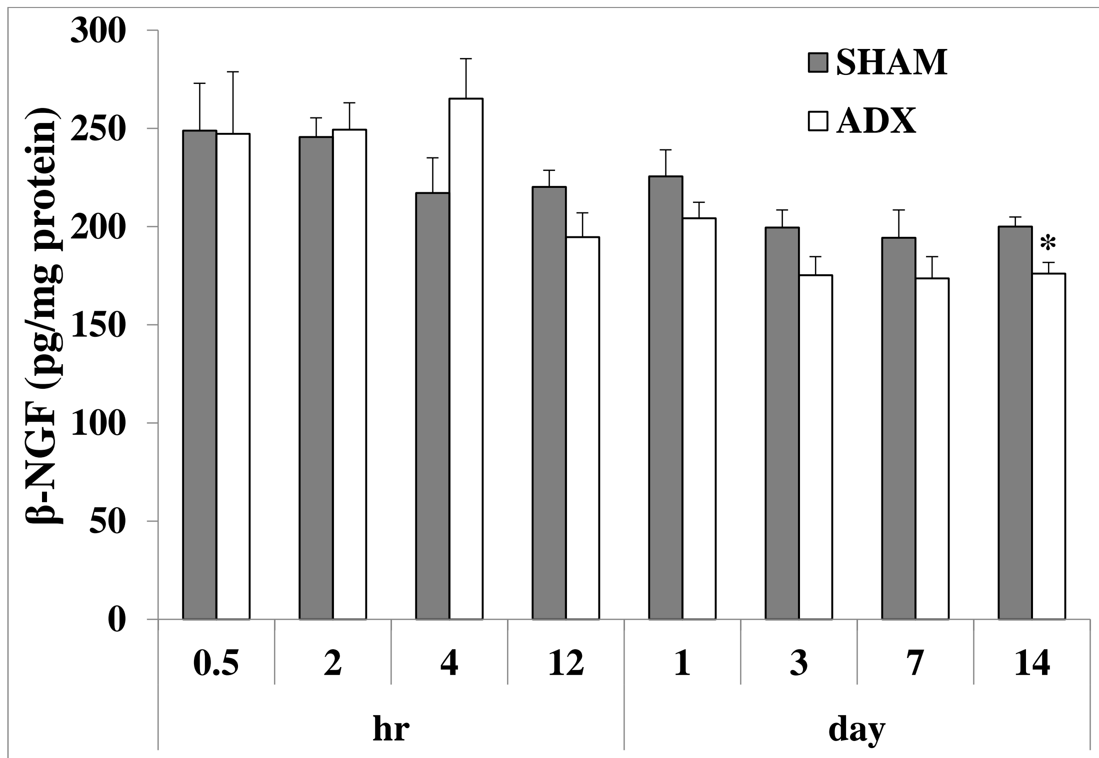

Figure 20: Bar graph showing β-NGF level in the hippocampus of adrenalectomized rats and sham operated rats. β-NGF level was measured by ELISA over the course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days). *$P<0.05$. Data are expressed as mean (±SEM)

3.4 Immunohistochemistry

To assess neurodegeneration due to ADX, we looked at microglia up-regulation, a major sign of cell death. We labeled the brain tissues of 1 day, 3 days, 7 days, and 14 days with a double immunofluorescent technique, using antibodies NeuN for neurons and Iba-1 for microglia.

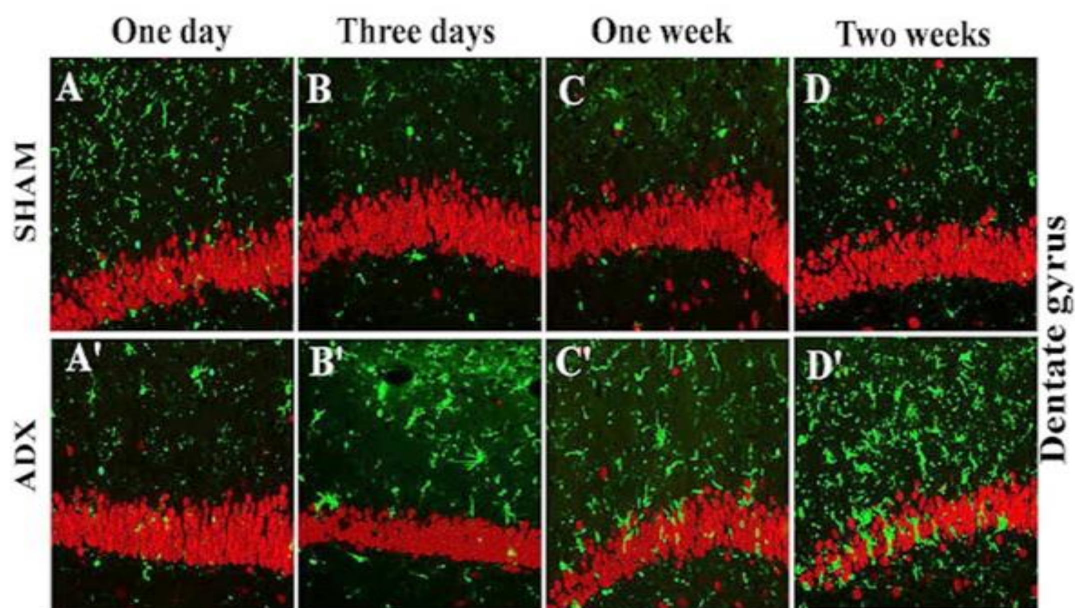

Figure 21: Images of coronal sections of the hippocampus stained with NeuN antibody (*red*) as a neuronal marker and Iba-1 antibody (*green*) as a microglia marker. The image shows the progression of microgliosis over the course of time (1 day, 3 days, 1 week, and 2 weeks) in the upper blade of the DG of the hippocampus of adrenalectomized rats compared to sham operated rats

The resulting images of coronal sections of the hippocampus show the progression of microgliosis over the course of time in the upper blade of the DG of adrenalectomized rats compared to sham operated rats. The outcomes of Iba-1 antibody staining did not show activated microglia in adrenalectomized and sham operated groups at day 1 after ADX (Fig. 21A' and A). At 3 days after ADX, in the dorsal blade of the DG of rats we detected activated microglia, whereas sham operated rats showed no sign of activated microglia (Fig. 21B' and B).

Iba-1 labeling exhibited activated microglia throughout the DG following 7 days ADX, this indicated a progression in microgliosis (Fig. 21C'). In contrast, sham operated rats; there was no staining of Iba-1 antibody in the DG of the hippocampus

at this time-point (Fig. 21C). At the 14 day time-point following surgery, the double immunofluorescence labeling was intensely evident in the DG of the hippocampus of ADX rats (Fig. 21D'), on the other hand, sham operated rats revealed no activated microglia (Fig. 21D).

In order to see the extent of the spread of the microglia over the whole dentate gyrus we labeled brain tissues of 14 days with an immunofluorescent technique using the antibodies Iba-1 for microglia. Our results show a total invasion of the whole dentate gyrus by microglia.

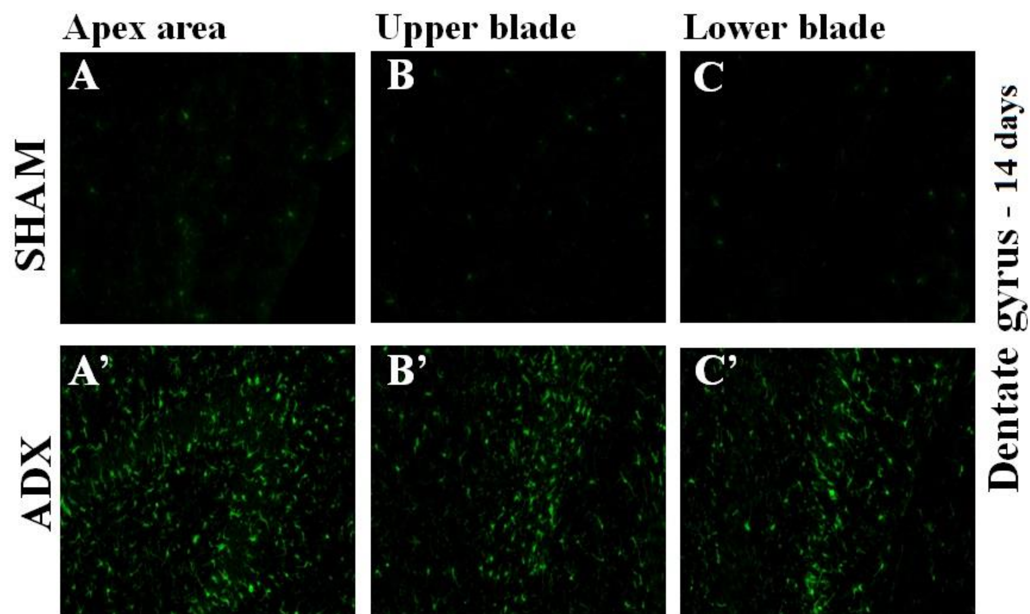

Figure 22: Images of coronal sections of the hippocampus stained with Iba-1 antibody (*green*) as a microglia marker. The image shows the extent of microgliosis at 14 days in the DG of the hippocampus of ADX rats compared to sham operated rats

Astrocyte activity in the hippocampus was studied at 1 day, 3 days, 7 days, and 14 days after ADX. The rat brain tissues were stained using immunofluorescent antibodies, NeuN for neurons and GFAP for astrocytes.

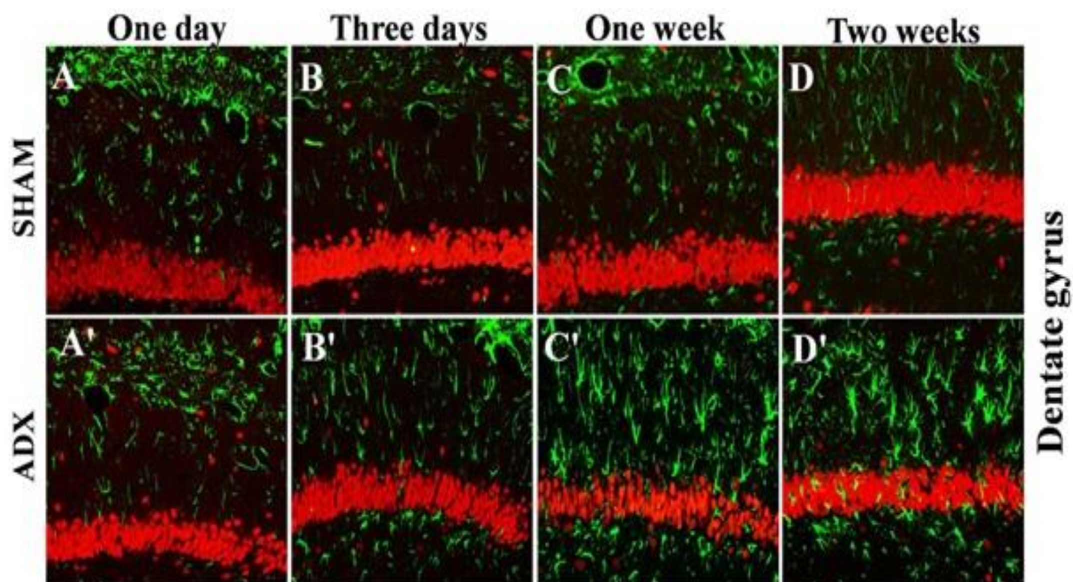

Figure 23: Images of coronal sections of the hippocampus stained with NeuN antibody (*red*) as a neuronal marker and GFAP antibody (*green*) as an astrocytes marker. The image shows the progression of astrogliosis over the course of time (1 day, 3 days, 1 week, and 2 weeks) in the upper blade of the DG of the hippocampus of adrenalectomized rats compared to sham operated rats

The images of coronal sections of the hippocampus show the progression of astrogliosis over the course of time in the upper blade of the DG of ADX rats compared to sham operated rats.

Importantly, the upper blade of the DG at 1 day and 3 day time-points did not show any observable change in the GFAP immunoreactivity between the hippocampi of adrenalectomized and sham groups (Fig. 23A' and 23B').

The activated astrocytes appeared at 7 days after ADX in the upper blade of the DG in the hippocampus (Fig. 23C') compared to the control (Fig. 23C). We were able to observe a strong immunoreactivity of GFAP antibodies in the hippocampus of 14-day adrenalectomized rats, with activated astrocytes appearing along the DG (Fig. 23D'). Sham operated rats did not show any astrogliosis at the same time-point.

In order to see the extent of the spread of the astrocytes over the whole dentate gyrus we labeled brain tissues of 14 days with an immunofluorescent technique using the antibodies GFAP for astrocytes. Our results show a total invasion of the whole dentate gyrus by astrocytes.

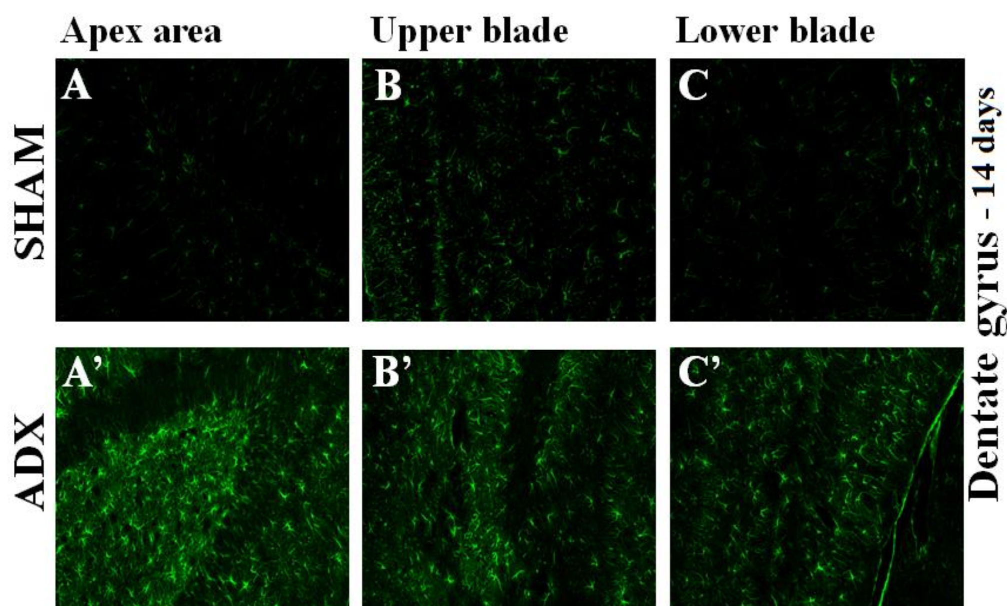

Figure 24: Images of coronal sections of the hippocampus stained with GFAP antibody (*green*) as an astrocytes marker. The image shows the extent of astrogliosis at 14 days in the DG of the hippocampus of adrenalectomized rats compared to sham operated rats

3.5 Oxidative Stress

The levels of oxidative stress markers MDA, GSH, and CAT in the hippocampal homogenates of adrenalectomized and sham operated rats were examined by ELISA over the course of time (0.5h, 2h, 4h, 12h, 1 day, 3 days, 7 days, and 14 days).

The levels of MDA were significantly increased in the hippocampus of adrenalectomized rats compared to bilateral sham operated rats after 7 days ($P<0.01$) and 14 days ($P<0.001$) of surgery (Fig. 25). However, at 0.5 hr, 2 hr, 4 hr, 12 hr, 1 day, 3 days, and 7 days before surgery no change was seen in the hippocampal levels of MDA between the two groups.

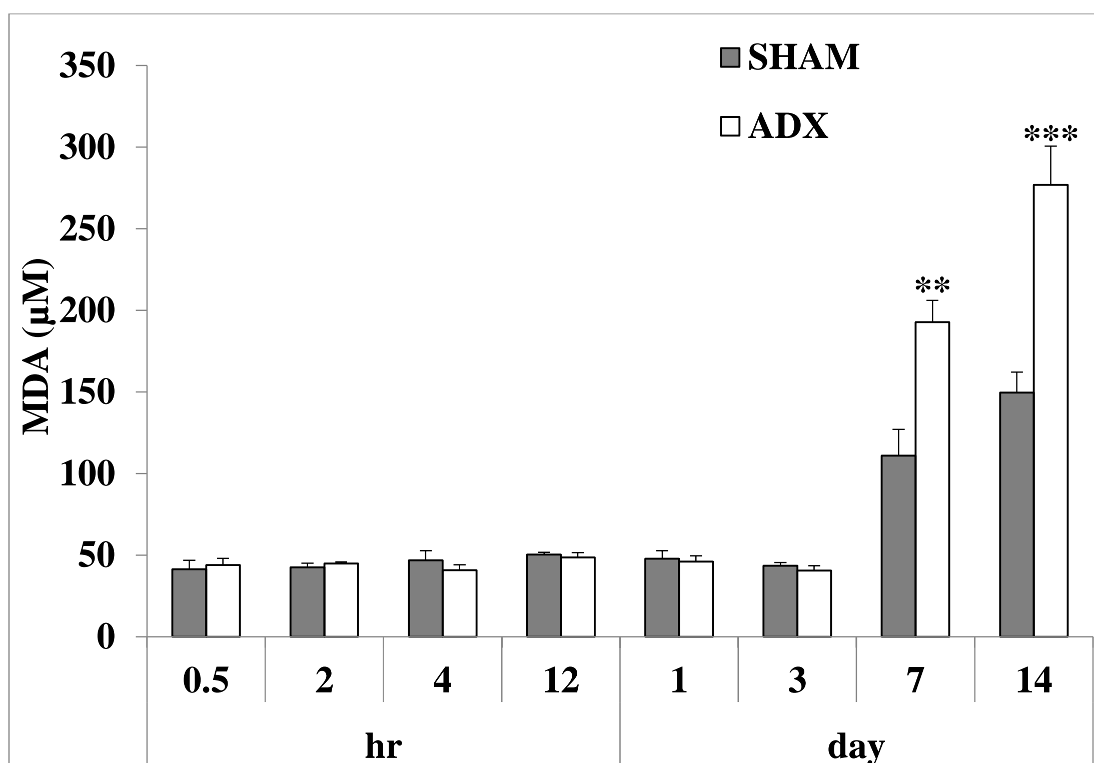

Figure 25: Bar graph showing MDA levels in the hippocampus of adrenalectomized and sham operated rats. Levels of MDA were measured by colorimetric assay over course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days) in adrenalectomized rats compared to sham operated rats. **$P<0.01$; ***$P<0.001$. Data are expressed as mean (±SEM)

A significant decrease in GSH levels was observed in the hippocampus 7 days (P<0.05) and 14 days (P<0.001) after ADX, however, no statistical difference in GSH levels were seen before one week (Fig. 26).

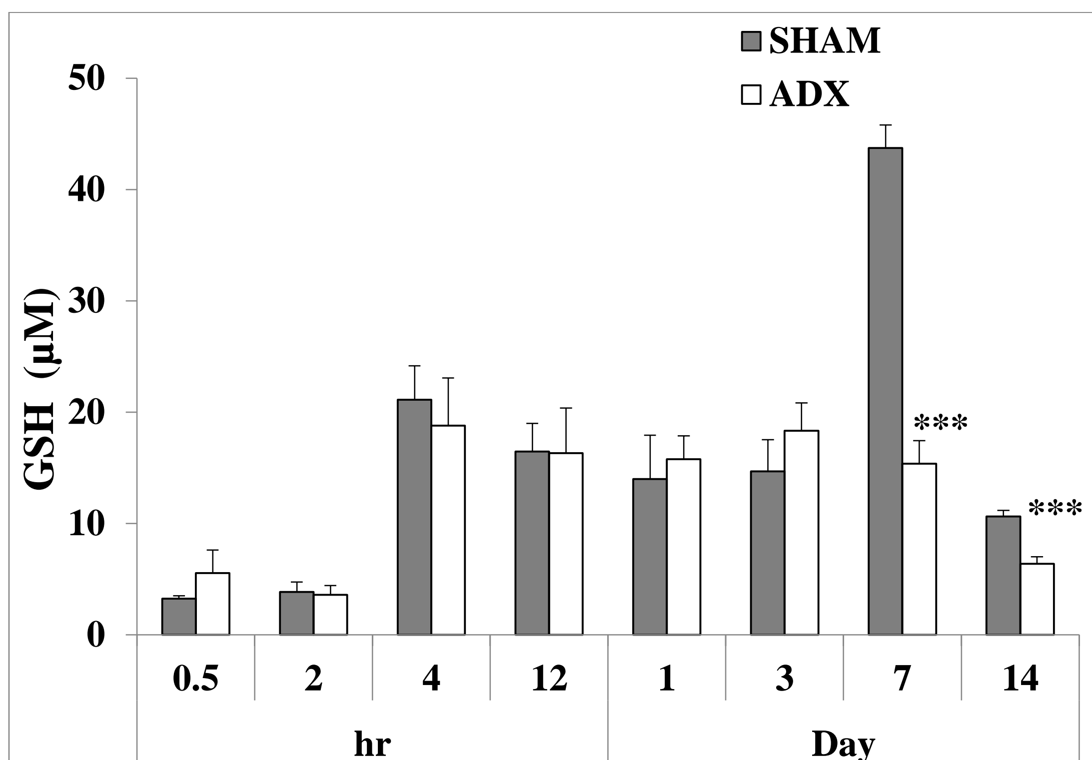

Figure 26: Bar graph showing GSH levels in the hippocampus of adrenalectomized and sham operated rats. Levels of GSH were measured by colorimetric assay over course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days) in adrenalectomized rats compared to sham operated rats. ***P<0.001. Data are expressed as mean (±SEM)

The levels of CAT were significantly increased in the hippocampus of adrenalectomized rats compared to bilateral sham operated rats after 3 days (P<0.01), 7 days (P<0.05), and 14 days (P<0.05) only (Fig. 27).

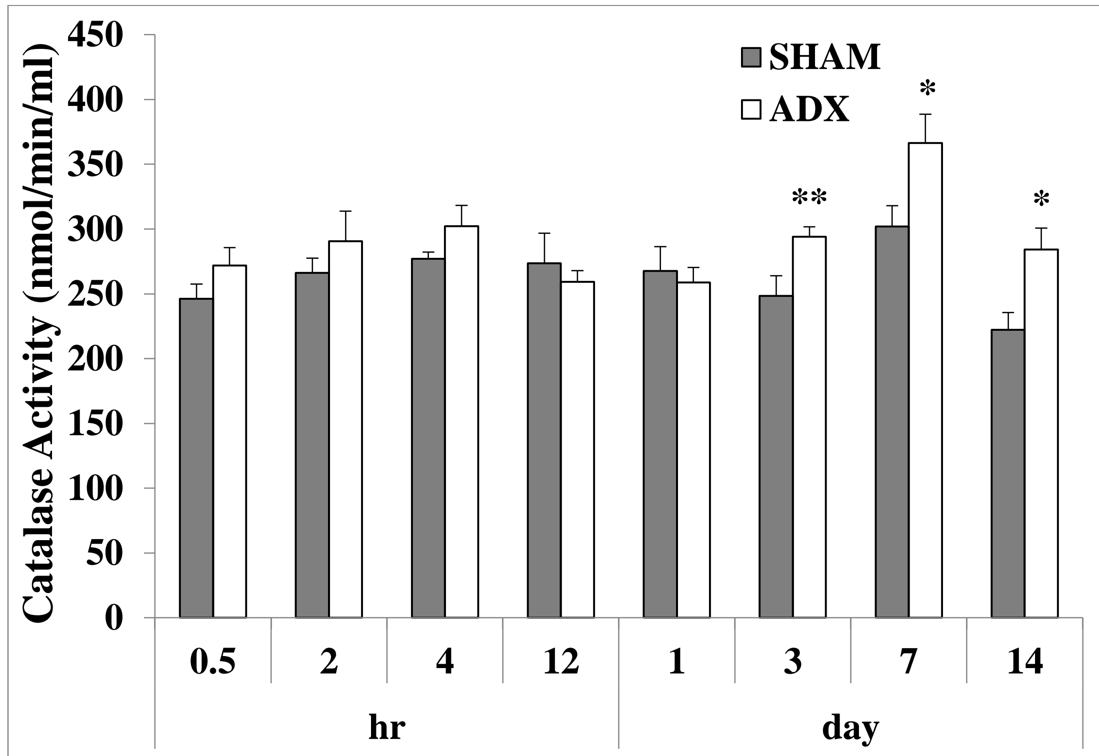

Figure 27: Bar graph showing CAT levels in the hippocampus of adrenalectomized and sham operated rats. Levels of CAT were measured over course of time (0.5 h, 2 h, 4 h, 12 h, 1 day, 3 days, 7 days, and 14 days) in adrenalectomized rats compared to sham operated rats. *P<0.05; **P<0.01. Data are expressed as mean (±SEM)

Chapter 4: Discussion

Bilateral adrenalectomy has been shown to damage the hippocampal neurons. Although the effects of long-term adrenalectomy have been studied extensively there are few publications on the effects of short-term adrenalectomy (Jaarsma et al., 1992; Vazquez et al., 1993; Hamadi et al., 2016). In this thesis we aimed to investigate the effects of short-term bilateral adrenalectomy on the levels of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α; levels of growth factors, the response of microglia and astrocytes to neuronal cell death as well as oxidative stress markers MDA, GSH, and CAT over the course of time in the hippocampus of Wistar rats.

Bilateral adrenalectomy significantly reduced serum CORT levels across all time points in rats compared to sham operated rats. These findings are in line with previous findings showing that bilateral removal of the adrenal glands in the adult rat produces a complete and immediate loss of CORT in the serum of ADX rats (Sloviter et al., 1989; Islam et al., 1999b). In control rats, high levels of CORT were seen early after the surgeries were performed, this is likely due to the stress generated from the procedure itself.

In this study we investigated the levels of inflammatory cytokines in the hippocampus of ADX and sham operated rats at different time points. Our results showed an early onset of pro-inflammatory cytokines elevation in the brain of ADX rats. IL-6 was significantly increased in hippocampus of ADX rats at 2 hours, 4 hours, 1 day, and 3 days. While, IL-1β levels were also significantly elevated after ADX at 4 hours, 1 day, 3 days, and 7 days. TNF-α was significantly increased in

hippocampus of ADX rats at a very early time point of 2 hours, 4 hours, and 12 hours. However, it appeared to be raised prominently at 14 days. In this study the early elevation of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α was observed prior to neuronal death. These findings are in line with several studies in which an early increase of different pro-inflammatory cytokines preceded neuronal damage (Minami et al., 1991;; Tchelingerian et al., 1993; Pearson et al., 1999; Rothwell, 2003; Liu et al., 2005; Zhu et al., 2006).

The involvement of IL-1β in neurodegeneration has been shown by different groups (Lawrence et al., 1998; Panegyres & Hughes, 1998; Rothwell, 2003). Mice or rats exposed to neurotoxins showed markedly exacerbated neurodegeneration when low doses of IL-1β were injected into the cerebral ventricles or brain parenchyma. Moreover, the co-infusion of IL-1β with IL-1β receptor antagonist inhibits the brain damage induced by the excitotoxin NMDA (Lawrence et al., 1998; Panegyres & Hughes, 1998). Furthermore, IL-1β is implicated in the pathology of several neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease and other CNS chronic diseases (Pearson et al., 1999; Allan & Rothwell, 2001; Boutin et al., 2001; Griffin & Mrak, 2002). IL-6 levels were also found to be elevated in different neurological diseases (Blum-Degen et al., 1995;;; Stelmasiak et al., 2001; Berti et al., 2002; Malmestrom et al., 2006; Lucin & Wyss-Coray, 2009).The early elevations of IL-1β, IL-6, and TNF-α observed after ADX might have a contribution to the initiation of the biological cascade responsible for subsequent hippocampal neuronal cell death.

Alteration of IGF-1 receptor and its messenger ribonucleic acid (mRNA) after long-term adrenalectomy were previously studied in the hippocampus by *in*

vitro receptor autoradiography and *in situ* hybridization immunohistochemistry, respectively. Significantly decreased levels of IGF-1 receptor and its mRNA were noted in the DG and CA1–CA4 regions of the hippocampus after long-term adrenalectomy suggesting that the level and expression of IGF-1 receptors in the hippocampus is influenced by adrenal hormones (Islam et al., 1998). In this thesis after short-term bilateral adrenalectomy of rats there was a significant reduction of IGF-1 levels in the hippocampus at 12 hours, 1 day, 3 days, 7 days, and 14 days. The level of IGF-1 was significantly reduced at 12 hours after ADX, this is seen long before the death of the neurons (day 3), and the appearance of the microglia (day 3) and astrocytes (day 7). However, the total loss of CORT and significant increase of inflammatory cytokines precedes the loss of IGF-1. Our results suggest that IGF-1 might have a neuroprotective role in the hippocampus. Interestingly, several studies have shown a rescuing effect of IGF-1 on cognitive functions in pathological conditions (Stern et al., 2014). It could be speculated that ADX may increase susceptibility of hippocampal neurons to degeneration through early changes in IGF-1 levels.

The level of β-NGF in young adult rat hippocampus was also found to be significantly decreased after ADX (Aloe, 1989). These findings are in line with our finding that β-NGF levels were significantly reduced in the hippocampus 14 days after ADX. It is possible that other trophic factors which have impact on hippocampal neurons might also be affected after ADX.

After bilateral adrenalectomy Sloviter et al (1989) performed Nissl staining of the rat hippocampus and revealed cell loss was bilateral and the neuronal degeneration first appeared at the lateral end of the upper blade of the DG (Sloviter et

al., 1989). These findings were replicated by several others (Sapolsky et al., 1991; Adem et al., 1994; Hamadi et al., 2016;). The cell death due to ADX occurs at approximately 3 days after the surgery was performed (Gould et al., 1990a; Spanswick et al., 2011; Hamadi et al., 2016). Interestingly, the lateral end of the upper blade of the DG where neuronal degeneration first appears is where the microglia and astrocytes appear after bilateral adrenalectomy. One of the major signs of neurodegeneration is the activation of microglia (Lucin & Wyss-Coray, 2009). We found significant increase of activated microglia around the dorsal blade of the DG 3 days after ADX. These findings support the observation by Gould et al (Gould et al., 1990b) which reported that cell death starts in the dorsal blade of the DG 3 days after ADX. Furthermore, our study shows that the number of activated microglia around the dorsal blade increased significantly one week after ADX and the whole DG was invaded by activated by microglia two weeks after the operation.

Increased astrocytes reactivity is associated with brain injury (Fiedorowicz et al., 2001). By increasing both their number and size astrocytes react to brain damage or disease. In this study we showed significant increases in GFAB immunoreactivity in the hippocampus at 7 days and 14 days after ADX. The molecular and polymorphous layers of the DG showed astrogliosis during the first week.

It is well known that the communication between astrocytes and microglia plays an important role in different pathological conditions (Verderio & Matteoli, 2001; von Bernhardi & Ramirez, 2001). Our results showed an early activation of microglia followed by activation of astrocytes on the third and seventh day respectively after ADX. Similar results were obtained in different neurodegenerative models where the microglia were first activated in response to neuronal damage

followed by astrocytes activation (Matsumoto et al. 1992; Frautschy et al., 1998; Gahtan & Overmier, 1999; Reali et al., 2005). Taken together these findings suggest that during neuronal damage there is a cross talk between microglia and astrocytes and that the former is considered as the driving force in recruiting the astroglia.

Accumulation or overproduction of ROS in conjunction with reduced antioxidant capacity within the cell results in oxidative stress (Sies, 1997). Microglia become functionally active as a result of neuronal death and up-regulate enzymes such as inducible nitric oxide synthase (iNOS) leading to an imbalance between free radicals production and the antioxidant defenses (Tran et al., 1997). One week after ADX an overexpression of iNOS in activated microglia revealing these cells as the source of the free radical in this model (Sugama et al., 2013). The antioxidant CAT was observed to increase at 3, 7, and 14 days in an effort to protect the neurons. However, a state of oxidative stress was manifested in the significant decrease of GSH 1 week and 2 weeks and simultaneous increase of MDA the indicator of the plasma membrane disruption 1 week and 2 weeks after ADX. These results are in line with the literature showing that several neurodegenerative models revealed the association between neuro-inflammation accompanied by reactive gliosis and a state of oxidative stress (Zhu et al., 2004).

The mechanisms underlying neuronal degeneration after bilateral adrenalectomy are not clear. The possibility that adrenocortical hormones might have direct effects on the survival of hippocampal neurons was previously suggested (Adem et al., 1994; Islam et al., 1999b). Hippocampal neurons might die when the mineralocorticoids (Type I) and/or glucocorticoid (Type II) receptors are not occupied by adrenocortical hormones (Adem et al., 1994; Islam et al., 1999a). Hu et

al reported that granule cell death after adrenalectomy was accompanied by the disappearance of GC receptor immunoreactivity in the granule cell layer (Hu et al., 1997). We have also previously suggested that the loss of adrenocortical hormones after ADX may cease to stimulate a factor(s), in the hippocampus, that is (are) necessary for the survival of hippocampal neurons (Adem et al., 1994; Islam et al., 1999b). In this respect our findings show early loss of IGF-1, before the start of neuronal degeneration and glial activation/migration, in the hippocampus after ADX. It could be speculated that ADX may increase susceptibility of hippocampal neurons to degeneration through early changes in IGF-1 levels.

4.1 Conclusions

Our study showed that short-term bilateral adrenalectomy leads to an early increase in pro-inflammatory cytokines and decrease in growth factors followed by neurodegeneration and activation of glial cells as well as oxidative stress. Taking these findings together it could be suggested that the early inflammatory components and loss of growth factors might contribute to the initiation of the biological cascade responsible for subsequent hippocampal neuronal cell death in the current neurodegenerative animal model (See Fig. 28). It could be speculated that in several neurodegenerative diseases like Alzheimer’s disease, chronic adrenal hormones increase and/or their receptors’ decrease may result in inflammatory mechanisms and loss of growth factors like IGF-1 which may lead to hippocampal neurodegeneration.

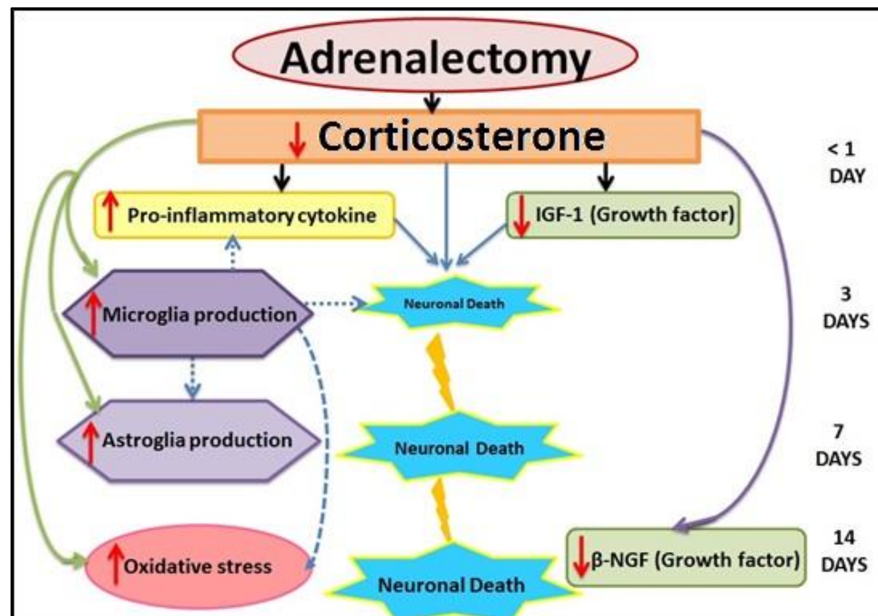

Figure 28: Cascade of neurodegeneration

4.2 Strengths and Limitations

Several strengths and limitations of our study should be recognized. A major strength of the study includes the well-performed ADX. No weight gain or death of subjects was seen. One limitation of our study is that the rats did not undergo spatial learning and memory performance tasks before or after experimental surgery which limited our ability to assess more specific associations between neurodegenerative alterations after short- term adrenalectomy and cognitive decline.

4.3 Future Studies

Future studies will be needed to investigate possible associations between memory performance and short-term bilateral adrenalectomy. Studying additional effects of ADX on the hippocampus are needed in order to understand the neurodegenerative processes involved in this animal model.

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