NEUROPROTECTIVE EFFECTS OF TUALANG HONEY SUPPLEMENTATION ON THE PREFRONTAL CORTEX FOLLOWING CHRONIC STRESS EXPOSURE IN RAT MODELS

BY

DR. FAIZAH BINTI MD NAWI

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE MASTER OF SCIENCE IN CLINICAL ANATOMY

UNIVERSITI SAINS MALAYSIA
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LIST OF ABBREVIATIONS AND SYMBOLS

µL	microliter
µm	micrometer
ACC	anterior cingulate cortex
ACTH	adrenocorticotropic hormone
AECUSM	Animal Ethics Committee of Health Campus
ANOVA	analysis of variance
ANS	autonomic nervous system
ARASC	Animal Research and Service Centre
ATP	adenosine triphosphate
β	beta
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
C	control
Ca	calcium
Cu	copper
Cl	chloride
CAT	catalase
CNS	central nervous system
CRH	corticosterone releasing hormone
CRL	Central Research Laboratory
CYP	cytochrome P450
df	degree of freedom
DNA	deoxyribonucleic acid
DPX	dibutyl phthalate in xylene
FAMA	Federal Agriculture Marketing Authorities
Fe	iron
FGF	fibroblast growth factor
FST	Force Swimming Test
GABA	gamma-aminobutyric acid
GAS	General Adaptive Syndrome
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter
GRs	glucocorticoid receptors
H	hydrogen
H_2O_2	hydrogen peroxide
HPA	hypothalamic pituitary adrenal
ST	stress + Tualang honey
Iba-1	ionized calcium binding adaptor -1

IFN-γ	interferon gamma
IL	interleukin
K	potassium
L-stage	locomotor stage
lPFC	lateral PFC
ml	milliliter
mPFC	medial prefrontal cortex
M-stage	mobile stage
Mg	magnesium
Mn	manganese
MRs	mineralocorticoid receptors
Na	sodium
NGF	neurotrophic growth factor
NG2-glia	polydendrocytes
ofPFC	orbitofrontal prefrontal cortex
O_2	oxygen
O_2^-	superoxide radical
$^1O_2$	singlet oxygen
$OH^·$	hydroxyl radical
pa	pascal
P	phosphorus
PBUH	peace be upon him
PFC	prefrontal cortex
PTSD	post-traumatic stress disorder
pH	potential for hydrogen
PVN	paraventricular nucleus
ROS	radical oxygen species
RO	alkoxy radical
RO_2	peroxy radical
RST	restraint stress test
S	stress
SAM	sympathomedullary
Se	selenium
SE	standard error
SEM	standard mean of error
SOD	superoxide dismutase
SNS	sympathetic nervous system
SPSS	Statistical Package for the social sciences
SRS	systematic random sampling
TB	tuberculosis
TH	Tualang honey
TGF β	transforming growth factor
T-stage	transitional stage
TNF-α	tumor necrosis factor alpha
Zn	zinc
α	alpha

KESAN PERLINDUNGAN SARAF DALAM PENGGUNAAN MADU TUALANG SEBAGAI MAKANAN TAMBAHAN TERHADAP KORTEKS PREFRONTAL BERIKUTAN PENDEDAHAN TEKANAN KRONIK KE ATAS MODEL TIKUS

ABSTRAK

Pengenalan: Tekanan telah menjadi perhatian utama dalam kalangan penyelidik kerana ia sangat berkait rapat dengan kesejahteraan psikologikal dan fisiologikal manusia. Pendedahan tekanan kronik menyebabkan pengaktifan berterusan pada paksi HPA yang mengakibatkan pengumpulan hormon kortisol dan radikal bebas sehingga menjejaskan banyak organ termasuk otak yang menyebabkan masalah penyakit berkaitan dengan tekanan. Madu Tualang ialah madu Malaysia yang berasaskan bunga-bunga liar, mengandungi kadar fenolik dan flavanoid yang lebih tinggi serta mempunyai aktiviti anti-oksidan dan anti-radang yang lebih kuat berbanding madu tempatan yang lain. Madu Tualang telah terbukti dapat melindungi otak daripada kerosakan oksidatif berpunca dari tekanan kebanyakkan melalui kajian neuron. Namun, kajian yang memberikan tumpuan kepada sel glial sokongan masih terhad.

Objektif: Objektif kajian ini dijalankan adalah untuk mengkaji kesan perlindungan madu Tualang terhadap astrosit dan sel mikroglial berikutan pendedahan terhadap tekanan kronik dalam korteks prefrontal model tikus.

Metodologi: Dua puluh empat ekor tikus jantan spesis Sprague Dawley dibahagikan kepada empat kumpulan: (i) kumpulan kawalan, (ii) kumpulan terdedah kepada tekanan, (iii) kumpulan madu Tualang (1.0g/kg berat badan dua kali sehari), (iv) kumpulan terdedah kepada tekanan yang dirawat dengan madu Tualang (1.0g/kg berat badan dua kali sehari). Kumpulan yang terdedah dengan tekanan dan kumpulan

terdedahkan kepada tekanan yang dirawat dengan madu Tualang diberikan dua jenis ujian tekanan berbentuk fizikal iaitu ujian sekatan pergerakan (RST) yang dijalankan selama lima jam dan ujian berenang secara paksa (FST) selama lima belas minit untuk 21 hari. Tikus dikorbankan pada hari ke 22. Otak dikumpul, di proses dan diwarnai dengan menggunakan teknik imunohistokemistri (IHC). Bilangan sel glial (astrocit dan sel mikroglial), bilangan proses utama sel glial, dan kepanjangan maksimum cabang sel glial dikira dan diukur menggunakan perisian imej Fiji J dan IC measure. Data analisis dijalankan dengan menggunakan perisian SPSS versi 26.

Keputusan: Kajian ini mendapati bilangan astrosit yang positif GFAP dan kepanjangan maksimum cabangnya telah berkurangan dalam kumpulan terdedah kepada tekanan berbanding kumpulan kawalan, manakala bilangan mikroglial yang positif Iba-1 dan kepanjangan maksimum cabangnya meningkat dalam kumpulan terdedah kepada tekanan berbanding kumpulan kawalan. Selain itu, bilangan astrosit yang positif GFAP dalam kumpulan terdedah kepada tekanan yang dirawat dengan madu Tualang meningkat berbanding kumpulan terdedah kepada tekanan, manakala bilangan mikroglial yang positif Iba-1 dalam kumpulan terdedah kepada tekanan yang dirawat dengan madu Tualang berkurangan dengan ketara berbanding kumpulan terdedah kepada tekanan. Juga, bilangan proses utama sel glial dalam semua kumpulan kajian tidak menunjukkan sebarang perbezaan.

Kesimpulan: Kajian ini telah menunjukkan bahawa madu Tualang sebagai makanan tambahan mempunyai kesan terhadap bilangan astrosit dan sel mikroglial, berkemungkinan melalui sifat antioksian dan anti radang yang dimiliki oleh madu Tualang. Namun, penggunaan madu Tualang tidak memberi kesan ke atas bilangan proses utama dan kepanjangn maksimum cabang dalam kedua-dua proses astrosit dan sel mikroglial selepas didedahkan dengan tekanan kronik.

NEUROPROTECTIVE EFFECTS OF TUALANG HONEY SUPPLEMENTATIONS ON THE PREFRONTAL CORTEX FOLLOWING CHRONIC STRESS EXPOSURE IN RATS MODEL

ABSTRACT

Background: Stress has been a focus of attention among researchers as it is closely related to human psychological and physiological well-being. Chronic stress exposure causes sustained activation of the HPA axis resulting in an accumulation of cortisol hormones and free radicals, affecting many organs including the brain, leading to the development of many stress-related disorders. Tualang Honey is a Malaysian wild polyfloral honey with higher phenolic and flavonoid contents and has stronger antioxidant and anti-inflammatory activities compared to other local honey. Tualang Honey has been shown to protect the brain against stress-induced oxidative damage dominantly in neurons. However, the studies that focus on the supporting glial cells are still scarce.

Objective: The objective of this study is to investigate the neuroprotective effects of Tualang Honey supplementations on astrocytes and microglial cells in the medial prefrontal cortex of rat models following exposure to chronic stress.

Methodology: Twenty-four male Sprague Dawley rats were divided into four groups: (i) control, (ii) stress-exposed, (iii) Tualang Honey treated group (1.0 g/kg body weight twice daily), and (iv) stress-exposed treated with Tualang Honey (1.0 g/kg body weight twice daily). The stress-exposed group and stress-exposed treated with the Tualang Honey group underwent two types of physical stress tests consisting of 5 hours of restraint stress test (RST) and 15 minutes of forced swimming test (FST) daily for 21 days. Euthanasia was performed on day 22. The brain was collected, processed, and stained using the immunohistochemistry (IHC) technique. The number of glial cells

(astrocytes and microglial cells), the number of primary processes of glial cells, and the maximum branch length of glial cells were counted and measured using Fiji J image and IC Measure software. Data were analysed, using SPSS software version 26.

Results: Present study found that the number of GFAP positive astrocytes and their maximum branch length processes were reduced in the stress group compared to the control group, while the number of Iba-1 positive microglial cells and their maximum branch length processes increased in the stress group compared to the control group. Besides, the number of GFAP positive astrocytes in the stress + Tualang Honey treated group increased compared to the stress group whereas the number of Iba-1 positive microglial significantly reduced compared to stress group. Also, the number of primary processes in both glial cells showed no significant differences in all study groups.

Conclusion: This study demonstrated that Tualang Honey supplementations has influenced the number of astrocytes and microglial cells in the medial prefrontal cortex possibly via its antioxidant and anti-inflammatory properties. However, Tualang Honey supplementation had no significant effect on the number of primary processes and the maximum branch length in both astrocytes and microglial cells processes after chronic stress exposure.

CHAPTER 1

INTRODUCTION

1.1 General Overview

In comparison to the past generations, stress has now become a pressing issue worldwide as it envelopes the lives of humans in innumerable ways. It has been the focus of attention among researchers for more than half a decade since it is closely related to human psychological and physiological well-being. Humans are undeniably living under continuous stress, oblivious to the fact that it may affect every aspect of our lives if not properly controlled.

For instance, the unprecedented Coronavirus COVID-19 outbreak recently has greatly impacted human mental health due to disease stigmatization, prejudice, and anxiety that has built up among individuals and society. This pandemic has forced more people to comply with mass quarantine as one of the national preventive measures in limiting the transmission of viruses. Regrettably, this quarantine is certainly detrimental to mental health, leading to the deterioration of social mental wellness (Javed *et al.*, 2020). As a result, this medical phenomenon is yet another contributing factor to the emergence of stress-related illnesses like anxiety, depression, post-traumatic stress disorders (PTSD), and Alzheimer disease (Godoy *et al.*, 2018). Additionally, based on the previous data, about 75 – 90% of stress related disorders are caused by overactivation of the pathophysiological stress system, demanding an

alternative therapy in bringing down the percentage to a much lower level. (Guidi *et al.*, 2019; YZ-Liu *et al.*, 2017).

Basically, stress can influence any systems in the human body including the nervous system, musculoskeletal, respiratory, cardiovascular, gastrointestinal, reproductive, endocrine, metabolic, and immune systems (Chu *et al.*, 2021). However, among all the systems, the nervous system particularly the brain is considered the key organ of stress resiliency and susceptibility because it is involved in the activation of stress anticipation, reactivity, recovery, and adaptation processes. Over the years, it is well accepted that enduring the stressor for a longer period is toxic to the brain as it may contribute to the induction of allostatic load or overload that is associated with poorer mental health outcomes (McEwen and Gianaros, 2010).

For the last five decades, the definition of stress is constantly evolving, refining, and progressing at so many levels, particularly in the study of health. To cover the broad definition of stress, Tsigos *et al.* (2020) have highlighted that stress is a threatened condition of homeostasis that is countered by adaptive processes comprising affective, physiological, biochemical, and cognitive-behavioural reactions in an attempt to preserve and restore balance. Simply, stress not only forces the body to absorb its damaging effects, but as well potentially can contribute to human mortality and it is depends on multiple factors such as type, duration, and severity of the exposure, as well as the individual perception and neurobiological vulnerability of stress (Yaribeygi *et al.*, 2017). Hence, based on these literatures, it is worth noting that both frequencies and the duration of stress exposure play a pivotal role in determining the extension of post-stress effects on human health.

To begin with, the human body is physiologically designed to adapt to acute stress since it is considered a basic requirement for human survival. Acute stress by

definition is a brief occurrence of specific threatening or difficult situations such as public speaking, job interviews, bumping into a snake, or catching a dateline for an assignment that clearly has a beginning and an endpoint. The mechanism of stress is basically composed of two systems; the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis that subserve as the basic component in the development of stress reactions and it is mostly determined by the duration of stress exposure. Acute stressor activates the ANS via sympathomedullary (SAM) pathways, resulting in the secretion of catecholamine hormones (adrenaline and noradrenaline) responsible for the development of the immediate actions or acute stress responses (Won and Kim, 2016). The elevated catecholamine hormones are the pathognomonic features in acute stress response (fight, fright, or flight) affecting the cell membrane of the smooth muscles as well as other parts of the body system resulting in the activation of the general sympathetic nervous system (SNS) reactions such as increased blood pressure, cardiac output, blood glucose levels and blood flow of the skeletal muscles preparing the body for serious physical activities in dealing with challenges (Chu *et al.*, 2021). Once the stimulus is turned off, the negative feedback mechanism leads to the restoration of homeostatic equilibrium. This is supported by a previous study, which found that temporary stressful conditions contribute to the differentiation of stem cells into new nerve cells, resulting in the improvement of mental performance in rat models (Qiao *et al.*, 2016). This indicates that acute stress has a beneficial effect on health not only in rats but also in humans.

In contrast to acute stress, chronic stress is caused by a series of repeated stressors that force the nervous system to fire continuously, resulting in the wear and tear of the body system. Most individuals who are subjected to chronic stress are unable to cope with it, resulting in an unresolved stress response that interfered with

the brain's homeostatic environment. This will lead to the development of various illnesses sequentially causing impairment of the overall quality of human life. At first, the mechanism of stress involved in chronic exposure is due to the constant release of catecholamines which later activates the HPA axis through the positive feedback mechanism, resulting in the secretion of glucocorticoid hormones known as cortisol. Serum cortisol at the optimum level is responsible for maintaining the alert state of the human body in response to persistent stressor exposure (Thau *et al.*, 2021a).

Unfortunately, recurring exposure to stressor can cause sustained activation of the HPA axis, resulting in excessive production of cortisol hormones which has catastrophic effects on the structural and functional integrity of many organs including the brain. Based on the previous studies, it has been established that over-secretion of cortisol hormones has been ostensibly recognized as the primary contributor to the pathogenesis of stress-related illnesses due to its detrimental effects of oxidative damage (Zhang *et al.*, 2021). Aside from that. decades of research have successfully proved there is a linear relationship between the duration of stress exposure to the structural and functional changes in the human brain. According to Yaribeygi *et al.* (2017), chronic stress exposure causes significant changes to the brain including alterations in neuronal histomorphology, decreased spine density, and causing neuronal plasticity due to the atrophied dendrites leading to the development or even exaggeration of a wide range of neuropathological disorders.

Other than the duration of exposure, the effect of chronic stress is also dependent on the capability of the human biological system to detoxify the end product of oxygen metabolism known as reactive oxygen species (ROS). Under normal conditions, most of the body's metabolic processes will lead to the generation of both free radicals and antioxidants. Both components work together in neutralising each

other in order to maintain the internal cellular environment. Unfortunately, persistent stress exposure trigger an imbalance between the production and detoxification of free radicals resulting in the formation of oxidative stress (Sharifi-Rad *et al.*, 2020). Uncontrolled oxidative stress results in the initiation of chronic and degenerative diseases such as accelerate ageing processes, as well as induce a variety of neurological pathologies such as Alzheimer disease, Parkinson disease, and depression (Mariotti, 2015).

Recently, there is an emerging trend among the worldwide population to search for natural resources as an alternative therapy in combating diseases. For those reasons, many researchers have shifted their focus to one of the most notable natural resources called Tualang Honey. Tualang Honey is one of the wild multiflora honey made by *Apis dorsata* species who keep their hives on one of the world's tallest trees called the Tualang tree which can be typically found in the tropical rainforest of Peninsular Malaysia (Fairuz Azman *et al.*, 2019). Based on their hive's location, Tualang Honey has been regarded by locals as one of the most treasured honey of all because it is made up of all kinds of exotic and unique flowers that are nearly impossible to be discovered outside the rainforest.

In general, honey has many therapeutic benefits such as antioxidant, anti-inflammatory, anti-microbial, anti-tumour, anti-mutagenic, and anti-diabetic properties (Sarfraz Ahmed *et al.*, 2018a). Other than mentioned properties, several studies have suggested that Tualang Honey may have anti-stress effects that should be investigated further in order to determine its preclinical potential in treating diseases especially stress-related disorders. For example, Azman *et al.* (2015) reported that administration of Tualang Honey can reverse the damage caused by noise stress exposure in rats and improve the pregnancy outcome in rats exposed to restraint stress

from day 11 of pregnancy until delivery, while according to the study conducted by Al-Rahbi *et al.* (2014) Tualang Honey supplementation has improved the serum cortisone level and adrenal cortex in stressed ovariectomized rats.

1.2 Justification and Benefit of the Study

After more than five decades of research on the baneful effects of chronic stress particularly on the brain, it is now the time to turn our efforts and attention towards the potential cure in combating the deleterious effects of chronic stress on human health despite the current available treatments. The demand for natural resources in the treatment of certain diseases has recently piqued the attention of most consumers owing to some promising potential effects seen in natural products like honey. It is also widely held by consumers that the use of natural products is safer because there is less chemical toxicity involved with fewer side effects (Silva *et al.,* 2021). As a result of this phenomenon, researchers around the world are expanding their research into the potential effects of honey, especially in treating and reversing the pernicious effects of chronic stress.

Anciently, honey has long been utilised for the treatment of many ailments due to its numerous medicinal properties (Eteraf-Oskouei and Najafi, 2013). One of the properties of honey that attracts researchers to broaden their scope in treating the effects of chronic stress is its antioxidant and anti-inflammatory properties. Both properties are the most powerful combination required in reversing chronic oxidative damage. According to the studies conducted by Bakar Sajak *et al.* (2020), Azman and Zakaria, (2019), Sairazi *et al.* (2018), Moniruzzaman *et al.* (2013) and Khalil *et al.* (2010) , Tualang Honey has higher total phenolic and flavonoid contents, as well as

stronger anti-radical scavenging properties, when compared to other local Malaysian honey such as Gelam honey, Pineapple honey and Borneo tropical honey.

While many studies have been conducted to investigate the protective actions of Tualang Honey on the neurons, studies that focus on the supporting cells of the brain, the glial cells, are still scarce. Therefore, the primary aim of this study was to investigate the protective effect of Tualang Honey on the brain supporting glial cells following exposure to chronic stress conditions in rat models. The present study focuses on the immunohistochemistry (IHC) method to identify the glial cells by their morphology.

1.3 Objective of the Study

1.3.1 General Objective

To determine the neuroprotective effects of Tualang Honey against chronic stress-induced changes in glial cells in the medial prefrontal cortex of a rat model.

1.3.2 Specific Objective

1. To compare the number of astrocytes in the medial prefrontal cortex between Tualang Honey supplemented rats following chronic stress exposure and related controls.
2. To compare the number of primary processes of astrocytes in the medial prefrontal cortex between Tualang Honey supplemented rats following chronic stress exposure and related controls.
3. To compare the average maximum branch length of astrocytes processes in the medial prefrontal cortex between Tualang Honey supplemented rats following chronic stress exposure and related controls.
4. To compare the number of microglial cells in the medial prefrontal cortex between Tualang Honey supplemented rats following chronic stress exposure and related controls.
5. To compare the number of primary processes of microglial cells in the medial prefrontal cortex between Tualang Honey supplemented rats following chronic stress exposure and related controls.
6. To compare the average maximum branch length of microglial cells processes in the medial prefrontal cortex between Tualang Honey supplemented rats following chronic stress exposure and related controls.

1.4 Research Question

1. Are there any significant changes in the number of astrocytes in the medial prefrontal cortex in Tualang Honey supplemented rats following chronic stress exposure compared to related controls?
2. Are there any significant changes in the number of primary processes of astrocytes in the medial prefrontal cortex in Tualang Honey supplemented rats following chronic stress exposure compared to related controls?
3. Are there any significant changes in the average maximum branch length of astrocytes processes in the medial prefrontal cortex in Tualang Honey supplemented rats following chronic stress exposure compared to related controls?
4. Are there any significant changes in the number of microglia cells in the medial prefrontal cortex in Tualang Honey supplemented rats following chronic stress exposure compared to related controls?
5. Are there any significant changes in the number of primary processes of microglial cells in the medial prefrontal cortex in Tualang Honey supplemented rats following chronic stress exposure compared to related controls?
6. Are there any significant changes in the average maximum branch length of microglial cell processes in the medial prefrontal cortex in Tualang Honey supplemented rats following chronic stress exposure compared to related controls?

1.5 Hypothesis of the Study

1. Tualang Honey supplementation prevents the changes of the astrocytes number in the medial prefrontal cortex following chronic stress exposure.
2. Tualang Honey supplementation prevents the changes in the number of primary processes of astrocytes in the medial prefrontal cortex following chronic stress exposure.
3. Tualang Honey supplementation prevents the changes in the average maximum branch length of astrocytes processes in the medial prefrontal cortex following chronic stress exposure.
4. Tualang Honey supplementation prevents the changes in the number of microglial cells in the medial prefrontal cortex following chronic stress exposure.
5. Tualang Honey supplementation prevents the changes in the number of primary processes of microglial cells in the medial prefrontal cortex following chronic stress exposure.
6. Tualang Honey supplementation prevents the changes in the average maximum branch length of microglial cells processes in the medial prefrontal cortex following chronic stress exposure.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Stress

Stress is a divisive terminology that has spawned a number of definitions and interpretations over the centuries. Stress is not a new term in the biomedical field, having been borrowed unintentionally from the engineering domain since the 1920s. In physics, it is defined as a measurable external force that causes a structural strain and it is quantified in pascal (pa) units. On the contrary, in biomedical field, there is neither a precise definition of stress with a fixed meaning nor a standard scale for evaluating stress due to the complexity of the stress itself (Epel *et al.*, 2018).

Historically, the foundation of stress research was laid and introduced in the early twentieth century by Walter Cannon and Hans Selye. Walter Cannon; an American physiologist coined the term homeostasis while introducing a breakthrough concept of "fight and flight response" in response to acute stress. Since the discovery, many researchers have been interested in expanding the scope of knowledge in the stress field until it was classified as the most established system explored in biological neuroscience (McEwen and Akil, 2020; McEwen and Gianaros, 2010). Later, the journey of stress continues to develop through an observation made by Hans Selye (father of stress research) when he was still a second-year medical student at Charles University in Prague, Republic of Czech. He was curious by the similar complaints such as loss of appetite, loss of weight, fatigue, low mood, and headache made by most of the patients who had been diagnosed with different diseases (Tan and Yip, 2018).

Hans Selye enthusiasm compelled him to initiate a discussion with one of his teachers, but he was ignored until in 1936 when he published his phenomenal article titled “A syndrome produced by diverse nocuous agents”. Based on his article, he defined stress as a “non-specific response of the body to any demand for change”. From there, he developed a concept called General Adaptation Syndrome (GAS) introducing 3 physiological phases of stress responses; (1) alarm stage, (2) resistance or adaptation stage, and (3) exhaustion stage. In his article, Hans Selye also has described his experimental findings found in the rat models when exposed to non-specific nocuous agents such as cold, surgical injury, intensive muscular exercise, and various drugs intoxication at sublethal dosages. He also discovered that the adrenal gland and pituitary gland were the prime endocrinal organs responsible for the generations of stress reactions (Godoy *et al.*, 2018; Fink, 2017; Selye, 1936).

Since the era of stress was introduced, many researchers including McEwen and Gianaros, (2010), Kim and Diamond, (2002) and Dhabhar, (2000) were actively involved in revising and expanding the context of stress. After nearly five decades of stress discovery and study, Mcewen and Stellar, (1993) had introduced a new paradigm of stress, proposing the concepts of allostasis, allostatic load, and allostatic overload. This concept emphasizes the relationship between stressors, individual vulnerability, and the cumulative impact of chronic stress on health.

2.2 Mechanism of Stress

Although it is true that stress has consistently been connected to the onset of many types of mental illnesses, it is equally true that stress can sometimes be forgotten as a part of normal physiological phenomena. The impact of stress on humans were dependent on several factors including the duration and severity of stress exposure as well as individual perception and neurobiological vulnerability to stress (Ebner and Singewald, 2017; Yaribeygi *et al.*, 2017). Acute stress is considered beneficial and necessary for humans since its response promotes focus, energy, and alertness when confronted with certain intense situations. However, chronic stress exposure causes exhaustion of the stress pathway which contributes to the development of many stress-related illnesses and at the same time causes exacerbations of pre-existing diseases (Lupien *et al.*, 2018).

Depending on the timing of exposure, stress is constantly interlinked with an activation of the ANS via the SAM pathway and the HPA axis. Physiologically, humans are subjected to adapt to temporary stress in their life as it acts as the key to human survival in case of threatening and difficult situations. Acute stress activates the acute stress response (fight, fright, or flight") in the brain stem via the sympathetic nervous system (SNS) resulting in the stimulation of chromaffin cells in the adrenal medulla and subsequently leading to secretion of the catecholamine hormones. These hormones are responsible for adjusting the body's physiology to the sympathetic reactions, allowing the body to respond appropriately to acute stress situations (Anderson *et al.*, 2019).

Chronic stress exposure, on the other hand, leads to activation of the HPA axis. HPA axis is involved in the interaction between three endocrinal glands that are responsible for maintaining the adaptive phase of the body against the physiological

changes in response to chronic stress exposure. It begins with stimulation of the paraventricular nucleus (PVN) in the hypothalamus and causes the production of corticotropin-releasing hormones (CRH). CRH is then released into the hypophyseal portal vessel leading to activation of the pituitary gland, resulting in the secretion of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH is the hormone responsible for stimulating the zona fasciculata in the adrenal cortex, which results in the secretion of the major glucocorticoid hormones known as cortisol (in human) or corticosterone (in rat) (Herman *et al.*, 2016; Sheng *et al.*, 2021).

2.3 Chronic Stress and Cortisol Hormone

Cortisol hormone plays a variety of roles in the human body, including in the inflammatory and immune responses, and are involved in the metabolism of glucose, lipid, and protein (Thau *et al.*, 2021b). The initial stress exposure causes the body to adapt to the physiological changes. When cortisol levels are still within the normal range, they act as a powerful anti-inflammatory agent by suppressing the release of substances that can cause inflammation. Physiologically, circulating serum cortisol exerts its functions by binding to the glucocorticoid receptors (GR) that are found in many organs and tissue such as the brain, heart, liver, lungs, stomach, uterus, muscles, and adipocytes. Among all, their receptor expression in the brain is the most critical and important area to be highlighted as its dysfunctionality carries catastrophic impacts on the overall of human mental health (Cruz-Topete and Cidlowski, 2015).

Under physiological conditions, cortisol binds to GR and acts as an anti-inflammatory agent. Long-term stress exposure, on the other hand, causes excessive secretion of cortisol hormones, resulting in the development of resistance towards their own binding sites. As a result, their affinity for GR is reduced whereas their affinity

for mineralocorticoid receptors (MR) is increased. Hence, once cortisol binds to MR, they act as pro-inflammatory agents instead of anti-inflammatory causing an increase in production of inflammatory cytokines such as IL1, IL6, IL8, IL12, IL18, tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) which sequentially destroys the GR and causing activation of microglial cells. Consequently, the disruption of GR results in malfunctioning of the negative feedback mechanism, leading to continuous release of cortisol hormones from the adrenal cortex (Hannibal and Bishop, 2014a).

In early 1920s, chronic stress has long been associated with the alteration of the immune responses due to prolonged activation of the immune system. In a study on the relationship between stress and immune response, Yaribeygi *et al.* (2017) mentioned that patients diagnosed with cancer and tuberculosis (TB) were among those who had been living under continuous stress. Previous research has suggested that one of the mechanisms supporting the occurrence of this phenomenon is due to morphological alteration of astrocytes, which leads to disruption of the blood brain barrier (BBB), allowing harmful stress mediators to breach the protective barrier and exacerbating the inflammatory response by provoking the immune system (Yaribeygi *et al.*, 2017; Hannibal and Bishop, 2014b). Other than that, chronic stress also has been reported to cause reduction in the production of antibody, increased the cytotoxic activity of lymphocyte by reducing the ratio between the helper/ suppressor T cells (40 – 70% lower than baseline); and involved in the modulation of cytokines secretion and lymphocytic trafficking (Chen *et al.*, 2015; Srivastava and Kumar, 2015).

The effects of cortisol at cellular level have been widely associated with the formation of free radicals or interchangeably known as reactive oxygen species (ROS). ROS are unavoidable toxic by-products released from the normal metabolism of

oxygen and biochemically they are unstable and highly reactive (Mittler, 2017). They are composed of superoxide radicals ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), singlet oxygen ($^1O_2$), peroxy radical (RO_2) and alkoxy radical (RO) (Li *et al.*, 2016). Under physiological conditions, the production and detoxification of ROS are counteracted and balanced by multiple anti-oxidants substances, derived either from enzymes or non-enzymatic scavengers such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, glutathione reductase, β-carotene (vitamin A), vitamins C and E and lipoic acids (Redza-Dutordoir and Averill-Bates, 2016).

Basically, non-toxic level of ROS are essential for the organisms because they play an important role as signaling molecules in the regulation of biological and physiological processes, for example in synthetising some cellular structures to fight pathogens during infestation (Mittler, 2017). However, a high concentration of ROS following persistent stress exposure always ends up with negative effects including damaging the cellular structures such as cell membranes, lipids, proteins, and DNA. Likewise, they are also involved in the prevention of major enzyme activation, normal cell division, and cellular process for the proper functioning of the human body (Pizzino *et al.*, 2017).

The activation of cytochrome P450 (CYP) in response to chronic stress exposure, leads to generation of cytotoxic ROS which reduces the radical scavengers (antioxidants) resulting in the formation of oxidative stress (Lewis, 2002). Uncontrolled oxidative stress thus contributes to the onset of many degenerative and neurological diseases including Alzheimer disease, Parkinson disease, depression as well as acceleration of ageing process (Sharifi-Rad *et al.*, 2020).

2.4 Stress Protocols in Animal Research

Rats are one of the most common research animals that are frequently used as a platform to study the effects of chronic stress exposure on human health. The study of chronic stress effect on health is impossible to be applied directly to humans since it is unethical and against human rights (Gambarana, 2006).

The selection of chronic stress test was mainly dependent on the direction of the study and the desired end-point effects. As for the current study, there were two types of chronic physical stress tests used to induce stress effects, namely restraint stress tests (RST) and forced swimming test (FST). Both tests were among the frequently used test in rats to elicit their physiological responses following chronic stress exposure. These methods are widely used due to their high throughput, applicability, low cost and good interlaboratory specificity and reliability. Furthermore, the implementation of both RST and FST tests have been shown to provide molecular and cellular changes including impairment of cognitive, affective, neuroendocrine and immune functions following the application of the test (Mo *et al.*, 2019b; Yankelevitch-Yahav *et al.*, 2015).

The duration of stress tests also plays an important role in determining the successful effect of stress on the rat models. The importance of the study duration was reflected in the research conducted by Sántha *et al.* (2016) and Imbe *et al.* (2012) who compared the effects of stress exposure over different time frame. The study showed that the number of astrocytes, the quantity of GFAP protein, and the intensity of GFAP fluorescence in the cerebral cortex of rats were significantly lower after 21 days of stress exposure, compared to application of stress on day 1 and day 3 (subacute). In addition, Hennebelle *et al.* (2012) discovered that 21 days of repeated stress test leads to activation of the HPA axis causing significant increase in serum corticosterone.

Hence, the stress duration for this study was set at 21 days.

Aside from the duration, the acclimatisation process is also necessary to be implemented prior to any animal intervention study. Based on UK Research Guidelines, transportation of the research animals is expected to cause mild to moderate stress which can be solved by a simple acclimatisation process. So, by definition, acclimatisation is a period of physiological, psychological, nutritional, and behavioural adjustment prior to being used in the research to restore homeostasis. The acclimatisation process allows the rats to become acquainted with new personnel, new environments and new settings (Conour *et al.,* 2006).

Referring to a previous protocol, both stress tests were done daily under random order of procedure. Randomization is the key success factor in any animal interventional experiment (Bebarta *et al.*, 2003). The application of daily randomization between RST and FST plays a pivotal role in this study because it helps reduce biasness between the rat models and prevent habituation throughout the experimental periods (du Sert *et al.,* 2020; Zamora-González *et al.*, 2013). Habituation is a confounding factor that must be eliminated because the prediction of repeated same stressor exposure can lead to physiological adaptation of stress response thus, resulting in the failure of HPA axis activation as well as the failure of hypothalamic CRH expression (Mo *et al.*, 2019a). The more randomization used in the studies, the more significant the data (Vesterinen *et al.*, 2010; Bebarta *et al.*, 2003). According to a meta-analysis data conducted by Hirst *et al.* (2014), non-randomized studies are more likely to produce exaggerated results which eventually affect the outcome of the study and lead to wasting of resources.

2.5 Animal Euthanasia

The main reference for the animal euthanasia method employed in this animal intervention complied with the most recent published American Veterinary Medical Association (AVMA) Guidelines which were also aligned with the protocol from Institutional Animal Care and Use Committee (IACUC) Leary and Johnson, (2020). Decapitation is a method of choice in this study because it allows for rapid loss of consciousness to the CNS without jeopardizing the structural integrity of the brain tissues and their perfusion. Although decapitation is aesthetically unpleasing, it is considered an appropriate method of euthanasia as it reduced suffering by hastening death (Hickman and Johnson, 2011). On top of that, decapitation also offers minimal to zero artifact creation in the tissue samples as it is free from contamination of any chemical agents (Shomer *et al.*, 2020). The guillotine was used to aid the decapitation method. The guillotine is widely in adult animal research settings because it can deliver a uniform manner of death, which was intentionally required in this experiment.

2.6 Introduction to Prefrontal Cortex

Apart from the heart, the brain is one of the most critical organs that require high oxygen supply to maintain its optimum functions. Due to its high physiological demand for oxygen supply, the brain is vulnerable to any forms of insults including chronic stress exposure which can lead to the formation of oxidative stress. Anatomically, the prefrontal cortex (PFC) is located in the frontal lobe, rostral to the motor and premotor cortices covering most of the ventral portion of the frontal lobe. The human PFC can be divided based on the topographical and functional divisions such as lateral PFC (lPFC), medial PFC (mPFC), and orbitofrontal PFC (ofPFC) (Kolk and Rakic, 2021).

The lPFC can be further divided into dorsolateral and ventrolateral. This area is the most important area in human as it is involved in the regulation of many executive functions including thought, planning, organising, maintenance of attention, reasoning, working memory, and decision making and also responsible for language comprehension (Arain *et al.*, 2013; Uytun *et al.*, 2018). Besides, anatomical evidences also have supported that the human lPFC was closely homologous to the medial PFC in rats (Seamans *et al.*, 2008).

The mPFC also can be subdivided into three areas; the infralimbic (IL), prelimbic (PL), and anterior cingulate cortex (ACC) (Hathaway and Newton, 2021). The mPFC on the other hand is the most affected area in the PFC as it is involved in the coping of stress responses. Hence, any prolonged insults associated with stress may modulate the structural and functional aspects of mPFC. Whereas the ofPFC is responsible for the control of emotion and cognition.

These functions of the PFC were first explored in 1848 inspired by the case of Phineas Gage, a famous American railroad construction worker. Phineas Gage is widely recognised as the man who began the era of neuroscience. He survived after a horrific accident being penetrated by a large tamping metal rod through his skull destroying big portion of his frontal lobe. Surprisingly, right after the injury, he was fully conscious and able to talk to his doctor while recalling the event on his own. However, after recovery, Gage underwent a dramatic personality changes. His character was completely changed by being stubborn, short-tempered, repulsive, and lack of consideration for others to the point where his friend said he was no longer Gage (Saladino *et al.*, 2021). Even after nearly two centuries, the case of Phineas Gage still continues to fascinate and inspire neuroscientists in exploring the complexities of the brain's functions, particularly the PFC.

The development of PFC began intrauterine as early as three weeks of gestation within the neural plate and continues postnatally until it reaches full development at the age of 25 years old (Arain *et al.*, 2013). The PFC, particularly the lPFC, evolved at a slower and prolonged pace in comparison to other brain regions and was noticed to be the last area that underwent complete maturation (Uytun *et al.*, 2018). As a result of its protracted development, any PFC remodelling requires special attention since its plasticity may remain across human lifespan. Therefore, during the developmental process, the PFC is expected to be the most vulnerable area to suffer from insults including chronic stress exposure.

In addition, the PFC is regarded as the central regulator of the brain due to its extensive connection with multiple neighbouring structures such as the hippocampus, hypothalamus, amygdala, basal ganglia, and other cortical areas. The vast connections made by the PFC are reflected in its functional complexities in processing higher-order executive functions (Arnsten, 2009). The basic mechanism in the functional regulation of the PFC is mediated by the recurrent excitatory connections between the neurons and supporting glial cells.

The PFC in both humans and rats plays a pivotal role in stress regulation and it has been attributed as one of the area most significantly affected by chronic stress, due to the presence of high glucocorticoid receptors and its direct involvement in the regulation of negative feedback mechanism in stress responses (Y. Z. Liu *et al.*, 2017; Lucassen *et al.*, 2014). According to prior research, the dendrites in the PFC area atrophied rapidly in less than one week after stress exposure, whereas the hippocampus region takes several weeks to show its damaging effects (Arnsten, 2009). This suggests that even minor and short-term insults can weaken the structure of the PFC, not to mention the long-term impacts of chronic stress exposure.

In the case of chronic stress exposure, excessive secretion of cortisol hormones was found to be the primary causes for the structural and functional impairments in the PFC. According to the previous findings, cortisol hormones cause cellular remodelling by reducing the length of cellular processes in the PFC, resulting in the disruption of neuro-glial transmission which contribute to the pathogenesis of stress-related disorders. Apart from that, the severity of mental-related illnesses appears to be directly proportional to the striking reduction in the numbers and dysfunctionality of neuroglial cells in response to prolonged stress exposure (Kaminsky *et al.*, 2016).

2.7 Histology of Prefrontal Cortex

Histologically, the PFC is mainly populated by neurons and multitype of glial supporting cells. There is a growing body of literature suggesting that the brain homeostasis is highly reliant on the functionality of neuroglial cells. Obviously, any modifications in the appearance, physiological dan functional properties of the neuroglial cells may significantly contribute to the pathogenesis of numerous mental-related disorders (Kaminsky et al., 2016; Czéh *et al.*, 2005).

Nowadays, despite being classified as supportive cells, research has revealed that neuroglia cells are as crucial as neurons. Glia is an ancient Greek word that means "glue," and it was originally thought to serve as the non-specific functional glue in the nervous system (Jäkel and Dimou, 2017a). In 1858, Rudolf Virchow, the founder of cellular pathology, proposed the term neuro-glia (nerve-cement) in his Cellular Pathology book as the true connective tissue supporting neurons or intercellular fibre that fills the space not populated by neurons (Oberheim *et al.*, 2012). Later, other researchers began to explore on the other possible functions of these cells which started in the middle of the 1860s. Stephen Kuffler, a forefather of modern neuroscience had

shifted his attention towards the function of glial cells refuting the theory that they were only supportive components of the nervous system, instead of serving as low electrical potentiate in response to potassium (Brown, 2017).

After more than a hundred years of discoveries, researchers globally agreed that the neuroglia residing in the central nervous system (CNS) can be divided into two major factions: macroglia and microglia. Macroglia were further classified as astrocytes, oligodendrocytes, and their progenitors, NG2-glia (polydendrocytes) (Verkhratsky *et al.*, 2019). Among the neuroglia divisions, astrocytes and microglia cells have been regarded as the most versatile cells where they were capable of experiencing substantial reactive modifications in response to their intercellular environment, particularly to chronic stress because they were the concern target for stress-induced changes (Pearson-Leary *et al.*, 2016; Matejuk and Ransohoff, 2020). Any dysfunctional phenotypic transformation of these two glial cells particularly in response to chronic stress contributes to the pathomechanism of stress-related disorders (Khakh and Sofroniew, 2015). Besides, these two populations of glial cells were among the central focus of gliobiological research because of their complex connections and their pathological activation has been connected to many forms of mental-related diseases (Kaminsky et al., 2016).

The proportion of the total glial cells to neurons and their distributions have been the most common debatable matter for centuries as none of these theories had solid scientific proof until now. Nonetheless, several concepts have been accepted that glia cells distribution varied among the species, and brain regions and it represent about 33 – 66% of total brain volume (Jäkel and Dimou, 2017a). The general histological structure of astrocytes and microglial cells includes their soma bodies, nucleus and many processes.

In a study conducted by Khakh & Sofroniew (2015), it was suggested that the terminology used in describing the processes of glial cells should be standardise as shown in Figure 2.1 and Figure 2.2. The primary processes (also known as branches or stem processes) are those that emerge directly from the soma bodies of the glial cells. The branchlets are the finer processes emanating from the branches and they are further subdivided into secondary, tertiary and higher order processes. Following the most recent data, the precise number of branchlets for each glial cell remains unknown (Khakh & Sofroniew, 2015). The very terminal extensions of glial cells branchlets are known as leaflets responsible for contact synapse

2.7.1 Astrocytes

It was widely agreed by researchers that astrocytes represent about 20 – 40 % of the total glial cells and regarded as the most abundant glial cells in the CNS (Qi *et al.*, 2019; Reemst *et al.*, 2016; Herculano-Houzel, 2014). The number of astrocytes residing in the CNS reflects the importance of their existence and explained their functional values more than serving as a supporter in the CNS. With regards to the numbers of their population, astrocytes have been recognised as the most well-defined glial cell in the CNS (Jäkel and Dimou, 2017b).

In 1893, Kolliker and Andriezen classified astrocytes into two different morphologies based on their location and complexity of the processes exhibited by the astrocytes known as protoplasmic astrocytes and fibrous astrocytes (Cunningham *et al.*, 2019). However, the illustration of the astrocytes was only first portrayed later in 1897 by Santiago Ramón y Cajal. While morphologically, astrocytes are characterised by star-shaped cells surrounded by multiple processes of different lengths that emerge directly from their soma body (Codeluppi *et al.*, 2021a). Astrocyte's processes are not