EFFECTS OF HYPOXIA EXPOSURE ON HUMAN HIPPOCAMPAL ASTROCYTES CULTURES

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EFFECTS OF HYPOXIA EXPOSURE ON HUMAN HIPPOCAMPAL ASTROCYTES CULTURES

By

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KESAN PENDEDAHAN HIPOKSIA TERHADAP KULTUR SEL ASTROSIT HIPPOCAMPUS MANUSIA

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LIST OF SYMBOLS AND ABBREVIATION

HhA	Human Hippocampal Astrocyte
AM	Astrocyte media
AGS	Astrocyte growth supplement
CNS	Central nervous system
IFs	Intermediate Filaments
P/S solution	Penicillin/ Streptomycin solution
DMSO	Dimethyl sulphoxide
GFAP	Glial Fibriallary Acidic Protein
HIF-1	Hypoxia Inducible Factor
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Bcl2	B-cell lymphoma 2
RT-PCR	Reverse Transcription Polymerase chain reaction
et al.	(et alia); and others

Abstrak

KESAN PENDEDAHAN HIPOKSIA TERHADAP KULTUR SEL ASTROSIT HIPPOCAMPUS MANUSIA

Otak memerlukan sumber oksigen yang berterusan untuk meneruskan funsi-fungsi kebiasaannya. Sebagai penguna oksigen yang terbanyak, otak sangat sensitif kepada hipoksia, iaitu keadaan kekurangan oksigen. Walaupun banyak kajian yang melibatkan haiwan menunjuk bahawa hypoksia menyebabkan kerosakan kepada neuron di hipocampus yang boleh mengundang defisit dalam pembelajaran dan memori, namun kecederaan terperinci yang disebabkan oleh hipoksia yang kronik di astrosit hipocampus manusia tidak diselidik lagi. Tujuan kajian kami adalah untuk memahami karakter sel astrosik hipocampus manusia yang didedahkan kepada keadaan hipoksik dan bagaimana perubahan tersebut berbeza mengikut tahap oksigen. Untuk kajian makmal, sel astrosik hipocampus manusia dan juga ruang hipoksia telah digunakan untuk meyamai keadaan hipoksik. Berdasarkan keputusan pemeriksaan awal, hampir 80% sel mati selepas 20 minit manakala 60% sel mati selepas didedahkan kepada kronik hipoksik,3% oksigen ($p<0.05$). Daripada data yang diperolehi, 15 minit telah dipilh sebagai titik tempoh kajian dan sel tersebut didedahkan kepada tahap oksigen yang berbeza. Analisis daripada ‘Trypan Blue viability assay’

menunjukkan hampir 15% sel mati setelah didedahkan kepada 15% oksigen, 25% sel mati dalam 10% oksigen, 48% sel mati dalam 5% oksigen dan 65% sel mati dalam 3% oksigen ($p<0.05$). Untuk 'immunofluoresce assay', GFAP telah digunakan sebagai penanda utama untuk menggambarkan morfologi sel astrosik. Mikroskop fluoresen mendedahkan filamen dan nukleus yang jelas dalam kumpulan sel yang tidak didedahkan kepada hipoksik. Sebaliknya, nuklei yang pecah disamping struktur sel yang rosak ditunjukkan dalam kumpulan sel yang didedahkan hipoksik kronik, 3% oksigen. Ekspresi GFAP di dalam lima kumpulan tersebut telah menunjukkan perbezaan intensiti GFAP. Perbezaan signifikasi purata intensiti GFAP yang ketara telah ditunjukkan didalam kronik hipoksik (p-value<0.001). Selain itu, HIF stain juga dilakukan untuk mengesahkan sel mati disebabkan keadaan hipoksia. Berdasarkan mikroskop fluoresen, perbezaan besar dalam ekspresi HIF-1α telah ditunjukkan dalam sel astrosik yang didedahkan hipoksik dan juga sel astrosik yang tidak didedahkan (p-value <0.001). Berdasarkan analisis molecular menggunakan RT-PCR, perbezaan signifikasi telah diperolehi dalam GFAP dan HIF-1α di dalam kronik hipoksia sel apabila dibandingkan dengan kumpulan kawalan dan akut hipoksik sel (p-value< 0.05). Sebagai konklusi, karakter sel astrosik mula berubah selepas didedahkan kepada kronik hipoksik, 3% oksigen selama 15 minit.

Abstract

EFFECTS OF HYPOXIA EXPOSURE ON HUMAN HIPPOCAMPAL ASTROCYTES CULTURES

The brain requires a continuous supply of oxygen to perform its normal function. Being the largest consumer of oxygen, it is especially sensitive to hypoxia, a condition in which brain receives reduced oxygen. Despite many animal studies reported that hypoxia caused neuronal damage in hippocampus which could deficits learning and memory, the exact damage caused by chronic hypoxia on human hippocampal astrocyte has not been analysed yet. Our aim for this study is to understand the characterization of human hippocampal astrocyte following hypoxia exposure and how the changes varied according to different concentration levels of oxygen. For the laboratory work, human hippocampal astrocytes cell line and hypoxia chamber were used in mimicking the hypoxic condition. Based on the preliminary screening, almost 80% of cell death occurred after 20 min and 60% cell death occurred in 15 min after exposed to chronic hypoxia, 3% of oxygen level ($p<0.05$). From the data gained, 15 minutes was chosen as the time point and the cells were exposed to different oxygen percentage. Analysis from Trypan blue viability assay showed about 15% of cells were

dead in 15% oxygen, 25% dead cells in 10% oxygen, 48% dead cells in 5% oxygen and 65% dead cells in 3% oxygen ($p<0.05$). For the immunofluorescence assay, a reliable marker Glial Fibriallary Acidic Protein (GFAP) was used in order to portray the architecture and morphology of astrocytes cells. Fluorescence scanning microscope revealed a filamentous and clear nucleas appearance in a control. In contrast, the rupture nuclei along with no rigid structure of cell were displayed in chronic hypoxia group, the 3% oxygen exposure. The expression of GFAP among the five groups showed different intensity of GFAP. The significant different of the mean intensity was clearly showed in the chronic hypoxia group (p-value<0.001). Along with that, the HIF-1 staining was performed to confirm the cell death due to hypoxia exposure. Based on the fluorescence microscope viewed, different expression of HIF-1α were displayed in all exposed astrocyte cells (p-value<0.001). In the molecular analysis using RT-PCR, there were significant changes of GFAP and HIF-1α in chronic hypoxia exposed cells when compared to control and acute hypoxia exposed cells (p-value< 0.05). To conclude, changes of the morphology of astrocyte cells are seen after 15 exposed to chronic hypoxia, 3% oxygen.

CHAPTER 1: INTRODUCTION

The brain requires a continuous supply of oxygen to perform its normal function. Regardless not performing mechanical work like skeletal muscle or heart, human brain is consider as one of the most metabolically active organ in the body. The brain utilizes 25% of the body's total oxygen consumption and expends about 3.5 ml of oxygen per 100 g of brain tissue per minute. The value needed for brain to function well in period of wakefulness remains constant even in sleep even though the rate of blood flow increased during sleep (Harris et al, 2012; Morselli et al, 2012; Hajjawi, 2014). As the largest consumer to oxygen, the brain needs oxygen desperately because it is important substrate to finely tune with signalling activities and cognitive functions (Ivanisevic and Siuzdak, 2015).

The brain also highly sensitivity to any significant changes in its environment, thus reduction of oxygen level may interfere with its optimal function. In general, hypoxia can be defined as reduction of oxygen level in cells or tissue of the body (Pighin et al, 2012). There are several critical components are involved in elucidate the hypoxia exposure, namely the rate of hypoxia occurrence, duration of hypoxia exposure, the present of reoxygenation and the severity of the hypoxic stimulus (Dempsey and Morgan, 2015).

In human brain, hippocampus is considered as one of the highly sensitive regions to hypoxia. Exposure to hypoxia would trigger several disastrous effects on the central nervous system (CNS), especially on the neurological and physiological aspects. Reduction of oxygen may also cause alteration in the brain structure (Ando et al, 2013; Mateika et al, 2015). For the molecular level, hypoxia condition may lead to elevation of free radical generation, oxidative stress and increased the level of L-type calcium channels (Barhwal et al., 2009; Hota et al, 2012). Other than that, hypoxia exposure may cause apoptosis or necrosis in hippocampal neurons which lead to impairment in learning and memory functions (Chiu et al, 2012). There are also growing body of evidences have clearly reported that alteration in oxygen level may lead to neurodegerative disorders and impaired cognitive functions in terms of learning and memory (Zhang et al., 2012; Smith et al., 2013).

Over the last few decades, the functions of astrocytes have been acknowledged widely in contributing to many essential functions in CNS. Apart from its numerous numbers that occupy the brain, astrocytes not only play a part as a number one supporting cell that provides its architectural structure as well as essential in anti-oxidant defence and inflammatory response. Besides, astrocyte cells support neuronal activity via astrocytic glycogen, induce neurogenesis from neural stem cells in the adult brain and acts as source of neural stem cells (DiNuzzo et al, 2012; Sirko et al, 2013).

Classically, astrocytes can be divided into two types according to their anatomical location and cellular morphology. Protoplasmic astrocytes dominant in grey matter while fibrous astrocytes located in all white matter (Sun and Jakobs, 2012). To characterise these specialized subtypes of astrocytes cells, several cells can be out lined such as Bergmann glia of the cerebellum and Muller glia of retina (Heller and Rusakov, 2015).

Astrocyte cells are involved in regulation of brain pathologies from acute lesions such as stroke and stress to chronic neurodegenerative processes such as Alzheimer diseases, Parkinson diseases and psychiatric diseases such as Schizophrenia and Bipolar disorder. Asides from its numerous essential functions in supporting neurons, they also involve in various activation programmes, which are important for; limiting the areas of damage, producing neuro-immune responses and for the post-insult remodelling and recovery of neural function (Kettenmann and Verkhratsky, 2011).

Activation of astrocytes cell, also known as astrogliosis arise as part of the response of the CNS to critical situation like neurotrauma, brain injury, ischemic damage and neurodegenerative diseases (Sofroniew and Vinters, 2010; Parpura et al, 2012). Regardless of its origin, the hallmark of reactive astrocyte is inflation of GFAP expression, vimentin and nestin. Generally, GFAP, vimentin and nestin can be categorized as building blocks of intermediate filaments (IFs), which form the cytoskeleton along with microtubules and actin filaments (Oberheim et al, 2012).

Immunohistochemical techniques that enable the detection of specific molecular markers at the single-cell level are essential tools for identifying and characterizing cells in healthy and pathological condition (Sofroniew and Vinters, 2010; Duraiyan et al, 2012). The astroglial component of gliosis is characterised by the accumulation of glial filaments, of which GFAP is the major constituent. Besides that, GFAP gene activation and protein induction appear to play a critical role in astroglial cell activation (O'Callaghan and Sriram, 2005; Yang and Wang, 2015). For decades, the expression of GFAP has been considered as the most reliable marker to immunohistochemically identify the astrocytes, even though not all astrocytes in the healthy brain express GFAP. GFAP also is not immunohistochemically detectable in all normal astrocytes, its expression exhibiting both regional and local variability. However, the use of antibodies to GFAP in histological studies has firmly established the existence of reactive gliosis as a dominant response to many different types of brain injuries (O'Callaghan and Sriram, 2005; Sun and Jakobs, 2012).

.Aside from GFAP marker, other astrocytes markers such as S100ß and glutamine synthetase have similar shortcomings (Sofroniew and Vinters, 2010 Oberheim et al, 2012). Recently, the aldehyde dehydrogenase 1 family, memer LI (Aldh 1L1, also known as 10-formyltetrahydrofolate dehydrogenase (FDH), was suggested as a pan-astrocyte marker used on transcriptome gene profiling and in situ hybridization (Cahoy et al, 2008; Sun et al, 2017).

CHAPTER 2 : LITERATURE REVIEW

2.1 Hypoxia

In the 21st century, the common causes of the death amongst average age men are heart infarction, stroke and cancer. There are many reasons on how these disorders conquer the human body, not only from environmental factors and lifestyle habits but also genetic predisposition. However, they all share a common feature in which the limitation of oxygen availability participates in the development of these pathological conditions. In molecular context, cells are able to cope with these threatening conditions like hypoxia conditions as they can trigger adaptive response to hypoxic conditions but their response are depending on the type of hypoxic condition either acute or chronic (Sjöberg and Singer, 2013; Kumar and Choi, 2015;).

Oxygen is the most vital element in maintaining the homeostasis and ensures efficiency of human body systems. Lacking of this chemical element or disruption of balance between its supply and demand may cause disparity changes. A condition where oxygen availability is limited can be described as hypoxia (Sjöberg and Singer, 2013; Nakazawa, 2016). In detail, hypoxia can be defined as the deficiency in the bioavailability of oxygen to the tissues of the body (Loiacono, et aI., 2010). Many situations where oxygen is lacking

not lead to death of the organism, or even cause damage but in extreme reduction of brain oxygen may lead to neuronal death. Deficiency of oxygen condition has been reported to trigger free radical generation and depletion of antioxidant status, thus leading to oxidative damage of vital cellular components (Rahal et al, 2014).

The brain is considered as the most hypoxia-sensitive organs because of its demand for a high oxygen supply, whereas the skeletal muscle is amongst the most hypoxia-tolerant. The brain, an organ with high metabolic rate along with a rich store of polyunsaturated fatty acids is count as the most sensitive organ and a vulnerable target to oxidative damage (Kalogeris et al, 2012). Indeed, the brain also has been categorized as one of the critical targets of stressors and act as the central organ which is responsible for stress responses, determining the adaptive or maladaptive responsiveness to various condition either acute or chronic. The architecture and structural function of the brain may disrupt due to its correspondent to the stressful events cause by stress, brain injury, oxygen deprivation or glucose alteration (Solaini et al, 2010; McEwen et al, 2012).

2.2 Different types of hypoxia condition

Brain needs approximately 20% of the oxygen consumed by the human body. The great demand of this oxygen is needed to produce ATP. ATP productions are required in order to maintain the membrane potentials which are necessary for electrical signalling of synaptic and action potentials (Harris et al., 2012). Interruption of this supply for more than a few minutes among most of vertebrates including human, may leads to irreversible neurological damage and neurological diseases. However, some studies reported that during mild hypoxia of short duration, the brain develops powerful neuroprotective and adaptive mechanisms that allow it to maintain normal physiological conditions (Rybnikova et al, 2012).

2.2.1 Maternal Hypoxia

Fetal stress such as hypoxia, malnutrition or excess glucocorticoids have a long lasting impact to the fetus development especially on developing brain. Exposure over a longer period to a pregnant mother may alter the fetal brain's ontogeny, organization, structure and functions (Harris & Seckl, 2011; Gonzalez-Rodriguez, 2014). Historically, Kingdom and Kaufmann in 1997 have suggested that hypoxic pregnancy condition can be divided into three; pre-placental hypoxia, uteroplacental hypoxia and post-placental hypoxia. Preplacental hypoxia can be described as a condition where both mother and her fetus would be in hypoxic condition. Usually pregnant mothers who live at high-altitude or having cyanotic maternal heart disease would experience this kind of condition.

Meanwhile, uteroplacental hypoxia is a condition where the maternal oxygenation is normal but the utero-placental circulation is impaired and the example where the situation implies is preeclampsia or placenta insufficiency. The post –placental hypoxia is a condition where only the fetus is in hypoxic condition. Lower level of oxygen during pregnancy is a one of common hostile environment associated with abnormal system of the pregnant mother which causes high risk to the fetus. Because the placenta helps to exchange oxygen, nutrients and waste between the mother and the offspring, therefore malfunction of placenta also may express acute and chronic effects on the developing fetus and drive to intrauterine growth restriction (IUGR), asphyxia, multiorgan failure and premature delivery (Herrera et al, 2014). Besides that, numerous animal studies have disclosed that maternal hypoxia affects the organogenesis of brain and heart (Tong et al, 2011; Davis et al, 2012).

2.2.2 Perinatal Hypoxia

Fetus in the utero does not undergo respiration process but imbalance of gas exchange due to defect of umbilical or uterine blood flow will lead to fetal asphyxia. The condition also can be described where there are low oxygen level in fetal blood and tissue decrease while the carbon dioxide level are high. The simultaneous changes of oxygen and carbon dioxide may lead to consequence of hypoxia condition which the injury effect to the fetal is depend on the time duration, intensity and occurrence rate of the insult (Baburamani et al, 2012; Thornton et al, 2012).

Hypoxia is considered as one of the most common causes of neonatal brain injury. However, it still remains to be well elucidated that why some brain regions are more sensitive in certain condition compare to other regions. Besides that, as the infant matures or after experience the insult, the susceptible of some brain regions are changed which may be due to on metabolic reserves and physiological adjustments (Rey-Santano et al, 2011). Post mortem studies pointed out that critical events such as hypoxic-ischemic and asphyxic episodes during pregnancy could lead to brain injury, morbidity and mortality. Cognitive impairment, delay in developmental progress, epilepsy, motor deficits and cerebral palsy also may occur due to intrauterine asphyxia (Glass et al., 2009; Baburamani et al, 2012).

One of the most common of brain damages among neonatal resulting from a shortage of oxygen or blood flow to the tissues is Infant Hypoxic-ischemic encephalopathy (HIE) (Stoll and Kliegman, 2007; Castillo and Chiang 2014). This major contributor to neonatal death and morbidity can be acute or subacute brain injury due to asphyxia and may occur prior, during or after birth. This kind of hypoxia-ischemia can be caused by placental insufficiency or infection, which also often an indication for preterm delivery by caesarean section (Goldenberg et al, 2008; Castillo and Chiang 2014). Epidemiological studies have showed that about 15%–20% of HIE cases died during the neonatal period and 30% of those who survive would develop neurological deficits and long-term neurodevelopmental disabilities in later life including, mental retardation, visual motor dysfunction, cerebral palsy and epilepsy neurodevelopmental disorders (Eghbalian and Monsef, 2008; Dauglass-Escobar and Weiss, 2012). As study of the pathophysiology of perinatal HIE is difficult to conduct

in human, a neonatal animal model known as Vannucci model has been used in numerous studies in mimicking the condition. In this model, 7-day postnatal rats experienced a unilateral common carotid ligation followed by systemic hypoxia in 8% oxygen balanced with nitrogen environment. The insult produces permanent hypoxicischemic brain damage limited to the cerebral hemisphere ipsilateral to the carotid artery occlusion (Vannuci and Vannuci, 2005; Riljak et al, 2016). Despite the advances in the last two decades in research of cellular processes and molecular mechanism underlying HIE, hypothermia could be the only effective treatment (Wu and Gonzalez, 2015).

2.2.3 Cerebral ischemia

The brain has homeostatic mechanisms which that can cope with mild ischemic attacks. However a severe ischemia overwhelms the mechanism resulting in cell death in smaller or larger parts of the brain. Cells that are experienced the ischemic condition may die within minutes or display delayed vulnerability depending on how early reperfusion is initiated, metabolic and ionic homeostasis can return and cell survival maintained. Basically cerebral ischemia disrupts several aspects of physiological, biochemical,genetics and molecular in human body which lead to malfunction of cellular integrity. Then, the alteration of the cellular mechanism may cause several consequences include imbalance of ionic level, glutamate excitatory, calcium overload and oxidative stress (Kalogeriset al, 2012; Bretón and Rodríguez, 2012).

There are two common types related to cerebral ischemia; global ischemia and focal ischemia. When cerebral blood flows (CBF) is reduced throughout most or all of the brain, this type of condition is known as Global ischemia. Meanwhile focal ischemia is explained by a reduction in blood flow to a very distinct, specific brain region (Lee et al, 2016).

2.2.4 Obstructive Sleep Apnea (OSA)

Sleep-disordered breathing is highly prevalent and it composes of several types which includes primary snoring, upper airway resistance syndrome, obstructive sleep apnea (OSA), central sleep apnea, and obesity-hypoventilation syndrome. The most common is the Obstructive sleep apnea (OSA), a disorder that is characterised by repetitive complete or partial obstruction of the upper airway during sleep which leads to decreased of air flow and snoring. Repeated apneic and hypopneic events during sleep would lead to intermittent hypoxemia, hypercania, cortical and sympathetic nervous system arousal and sleep fragmentation (Yadav et al, 2013; Baril et al, 2015). OSA is commonly related to diminish neurocognitive function, neuropsychological impairments and cardiovascular morbidities. OSA would affect the sleep fragmentation which later reduced quality of life, impaired work-performance and also increased risk of motor vehicle accidents in most cases (Yadav et al, 2013; Garvey et al 2015).

However, in a worst case scenario, this type of sleep disorder may change directly to several part of brain function especially on cognitive function and cause the neurobehavioral consequences (Beebe, 2011).

Previous studies have been focused to investigate the specific cognitive functions and some have attempted to identify a "pattern" of cognitive dysfunctions in OSA. Executive functioning, a set of mental skills which include essential function in planning, initiation, execution of goal-oriented behavior and mental flexibility, is another affected domain. Indeed, several studies insisted that it is the most prominent area of cognitive impairment in untreated sleep-disordered breathing which can be found both in adults and children (Zimmerman et al, 2012; Olaithe et al, 2015; Krysta et al, 2017).

2.2.5 High altitudes sickness

As the altitudes location getting higher, the barometric pressure is reduce, thus less oxygen is inhaled. This kind of situation where oxygen availability is restricted may lead to imbalance of oxygen in brain tissue and cause cerebral damage, neurological deficits and cognitive dysfunctions. Today's increasing popularity and ability to travel rapidly to high altitudes exposed millions of people to acute mountain sickness (AMS), a most common condition occur in those that go too high and too fast. Typically, AMS will be experienced by non-acclimatized mountaineers or high-altitudes training's athletes within 6-12 hours of arrival to altitudes above 2,500m (Bärtsch and Swenson, 2013; Netzer et al, 2013). The symptoms include dizziness, anorexia, nausea, vomiting, fatigue and insomnia.

AMS also is considered as mild High-altitudes cerebral edema (HACE), a severe case that may face by the climbers above 4,000 m. HACE is characterized by altered consciousness, ataxia, or both in a subject with AMS (Bailey et al, 2009; Netzer et al, 2013). HACE can progress quickly, usually from mild ataxia to a coma, with death occurring within hours. If remains untreated, HACE can cause brain herniation from unchecked cerebral edema.

Besides that, a rapid ascent to high altitudes also can lead to potentially fatal consequences, known as High-altitude pulmonary edema (HAPE). HAPE is one of the causes of most death related to high altitudes. HAPE also hard to diagnose earlier as it showed common symptoms for climbers such as shortness of breath, tachypnea, tachycardia, reduced arterial saturation, fatigue, and cough. The onset of HAPE is usually delayed and typically occurs 2–4 days after arrival at altitude (Darosa et al, 2012; Derby and deWeber, 2010).

2.3 Vulnerability of brain to hypoxic

Brain tissues continuously demand oxygen even in inactive state, unlike most other tissue in human body. Even though its weigh is less than 2% of body mass, but it still needs an impressive oxygen supply as the small tissue mass is required to support the high rate of adenosine tri-phosphate (ATP) production in order to maintain an electrically active state for the continual transmission of neuronal signals.

Corresponding to anoxia, a condition of zero oxygen will cause the ATP fall drastically within minutes which lead to highly destructive consequences. Other complications that made the scenario worst include stroke, head trauma, brain injury and others (Mergenthaler et al, 2013). The injuries continue to worse and become irreversible in prolonged exposure except re-oxygenation is restored. Mainly necrosis is the reason of the acute cell death occurs but hypoxic condition also trigger delayed apoptosis (Chavez-Valdez et al, 2012).

Among different parts of brain, the most susceptible regions to hypoxic insult are the cerebral cortex, hippocampus and sub-ventricular zone. To be specific, hippocampus has been indicated to be more vulnerable to hypoxia stress compared with the cerebellum and cortex (Jai et al, 2013). In the hippocampus it was reported that a reducing essential elements like oxygen induced neuronal death in the CA1. Fetal brain injury due to maternal hypoxia may associate with inflammation and cause imbalance of protein or hormones. Exposure of chronic hypoxia cause elevation of lactate:pyruvate ratio and decrease of the GSH:GSSG ratio, a favorable pro-oxidant state. Besides that, there were arising of expression levels of some pro-inflammatory and several pro-apoptotic proteins including Bax, Bcl-2 and p53 (Guo et al, 2010; Wang et al, 2016).

2.4 Hypoxia in different species

In mammals or human specifically, brain function is vulnerable to the effects of hypoxia and in some condition, it can be irreversibly impaired by even brief periods of low oxygen supply. In biological aspects, the cellular ATP demands of most mammalian cells and tissues remained constant even though the oxygen supply is reduced. This kind of situation may lead to energetic deficit that can be made up for only by activation of anaerobic ATP supply pathways, the Pasteur Effect. However, because of the rapid depletion of fermentable substrate together with the accumulation of deleterious end-products, this anaerobic pathway unable to cope with the pre-existing energy demands. The failure in fulfil the oxygen demand resulting in necrosis and cell death (Thornton et al, 2012; Hagberg et al, 2014)

However, in certain vertebrate species, they acquired to cope with brain hypoxia as the availability of oxygen is limited in their natural environments. Four model hypoxia tolerant species includes freshwater turtles that can survive several months trapped in frozen-over lakes, coolest burrows of arctic ground squirrels hibernated at extremely low rates during winter, seals and whales diving mammals that can undertake breath-hold dives up to several hours without signs of deleterious hypoxia effects and naked mole-rats that faced hypoxia condition in their entire life as they lives completely in underground (Schneuer et al, 2012; Larson et al, 2014).

These remarkable specializations of brain physiology shown by these species were essential for them to survive acute or chronic episodes of hypoxia. To be specific, these species are able to adapt the hypoxia condition because of their body morphologies, habitat and utilization of dormancy (Schneuer et al, 2012; Jonz et al, 2015). Body system acquired by these species may be differ from human but deep understanding on how it co-operate in order for hypoxia survival are really important as it can lead to better appreciation of how nervous systems are adapted for life in specific ecological niches. Besides that, analyses on these systems may become crucial elements that can be applied in therapy for neurological conditions such as stroke and epilepsy (Larson et al, 2014; Jonz et al, 2015).

2.5 Morphological and types of astrocytes cells

Glial cells were first described by Virchow in the middle of the nineteenth century as a merely supportive structural element of the nervous system. The astrocytes comprise a heterogeneous family of morphologically and functionally distinct cells whose structural plasticity is maintained mostly by a filamentous network consisting mainly of vimentin and GFAP (Yang and Wang 2015). Astrocytes, a star-shaped cells are distributed throughout the brain and spinal cord. The term astrocytes itself derived from combination Latin word for stars, (*astra*, singular *astrum*) and *cyte,* which is in turn derived from the Greek word *kytos,* meaning vessel.

Historically, in 1858 Rudolf Virchow was the first researcher who proposed that neuroglia comprised the connective tissue of the brain and the cellular elements complete them. Later, the present of astrocyte in the CNS was described by Camillo Golgi who then furthers wider the concepts that these cells are the "glue" of the brain. In 1893, Michael von Lenhossek introduced the term 'Astrocyte'. After that, Koliker and Andriezen categorized astrocyte cells into two; fibrous and protoplasmic astrocytes. Then, the extraordinary pleomorphism of astrocyte cells was visualized by Ramón y Cajal (Kimelberg and Nedergaard, 2010; Oberheim et al, 2012; Chaboub and Deneen, 2013).

Morphology of astrocytes is diverse in character. Numerous studies related to these diverse neuroglial cells have shown that these cells are not uniform and have many unequivocal definitions (Kimelberg and Nedergaard, 2010; Lee and MacLean, 2015). Some astrocytes do portray a star-like shape with several stem processes originating from soma. Meanwhile, some of them do not display the star-like shape and not contact brain capillaries. In addition, not all of them express the glial fibrillary acidic protein (Hewett, 2009; Placone et al, 2015). Glial fibrillary acidic protein (GFAP) is one of the major astroglial intermediate filaments beside vimentin which are form of cytoskeleton that expressed the structural of astrocyte architecture. It also considered as one of the specific marker in differentiate astrocyte cell from others (Placone et al 2015).

Classically, these cells are divided into two; protoplasmic and fibrous type depending on their morphology and their location in central nervous system (CNS) (Privat and Rataboul 1986). Protoplasmic astrocytes are commonly found in grey matter while the fibrous astrocyte dominated the white matter (Placone et al, 2014). Protoplasmic astrocytes have numerous complex fine processes which contact blood vessels and form a 'perivascular' endfeet and some of them send processes to the pial surface to form the 'subpial' endfeet. These types of astrocytes also form multiple contacts with neurones. In contrast to protoplasmic, the processes of fibrous astrocytes are long and less complex. Several perivascular or subpial endfeet are established and numerous extensions were sending through these fibrous processes in order to contact axons at nodes of Ranvier (Oberheim et al, 2012; Placone et al, 2014).

Larger number of astrocytes has made these cells as the most important supporting cell in brain. These cells exclusively tile the entire CNS and provide many important functions in order for brain to function well. In respond to all forms of CNS insults, reactive astrogliosis will be triggered which is consider as a pathological hallmark of CNS structural lesions (Sofroniew and Vinters, 2010; Parpura et al, 2012). Neuropathologist such as Carl Frommann, Franz Nissl, Alois Alzheimer and Pio del Rio-Hortega recognized the pathological potential of astrocyte cells at the end of the 19^{th} to the beginning of the 20^{th} centuries. In spite of that, the particular about neuroglia and astroglia remains incomplete because of a long-lasting prevalence of neurocentric views in neurology and neuropathology.

Table 2.1 Types of astrocytes

Types of astrocytes	Location	Details	References
Protoplasmic astrocytes	Abundant in grey matter and highly heterogenous between and within the brain.	Have bushy appearance with thick and branches processes. Protoplasmic astrocytes have extends 1-5milimeter long processes which terminate either in the neutrophil or on the vasculature.	Oberheim et al, 2009; Sofroniew et al, 2010; Oberheim et al, 2012
Fibrous astrocytes	Localized in white matter and less complex than protoplasmic astrocytes.	Star-shaped cells. Have long fiber-like, thin and straight processes Fibrous astrocytes have numerous finger-like outgrowths or known as perinodal processes that contact axons at nodes of Ranvier.	Wang and Bordey, 2008; Sun and Jakobs, 2012
Interlaminar astrocytes	Small glial cells, resides in upper upper cortical layer. Extends their	Spherical cell bodies and contained several short processes that extend in all	Oberheim et al, 2009; Oberheim et al, 2012; Robertson, 2014

	GFAP positive processes through cortical layer 2-4.	directions that contribute to the pial glial limitains, creating a thick network of GFAP fibers.	
Varicose projection astrocytes	Reside in the 5^{th} and 6^{th} layer of the cerebral cortex.	Exhibit 1 to 5 long processes and characterised by evenly spaced varicosities. These type of astrocytes has not been not describes in any infraprimate species. The processes of the varicose projection astrocytes did not respect the domain organization, as they traveled in all directions, piercing and traversing the domains of neighboring protoplasmic astrocytes.	Oberheim et al, 2012; Tabata, 2015
Bergmann glia	Specialized radial glial in cerebellum, reside in the Purkinje-cell and the granular layers of the Cerebellar cortex.	Have long processes extending towards the molecular layer of the cerebellar cortex, exhibit the pial vascular endfeet.	Bellamy, 2006; Marzban et al 2015

Fananas cells	In the molecular layer of the Cerebellar cortex.	Have several short side processes and feather-like of cellular morphologies.	Şovrea and Bosca, 2013; Chandrasekaran et al, 2016
Müller cells	Predominant glial in retina.	Types of radial cells and have an intense metabolic activity. Muller cells also contain microfilaments and glycogen within their cytoplasm.	Şovrea and Bosca, 2013; Chandrasekaran et al, 2016; Vecino et al, 2016
Pituicytes	In the neurohypophysis	Irregular shaped cytoplasm and their cytoplasm contains lipid droplets and pigment granules.	Chandrasekaran et al, 2016
Inerstitial epiphysial cells	In the epiphysis	Exhibit cytoplasmic processes and contain numerous filaments within their processes.	Şovrea and Bosca, 2013; Chandrasekaran et al, 2016

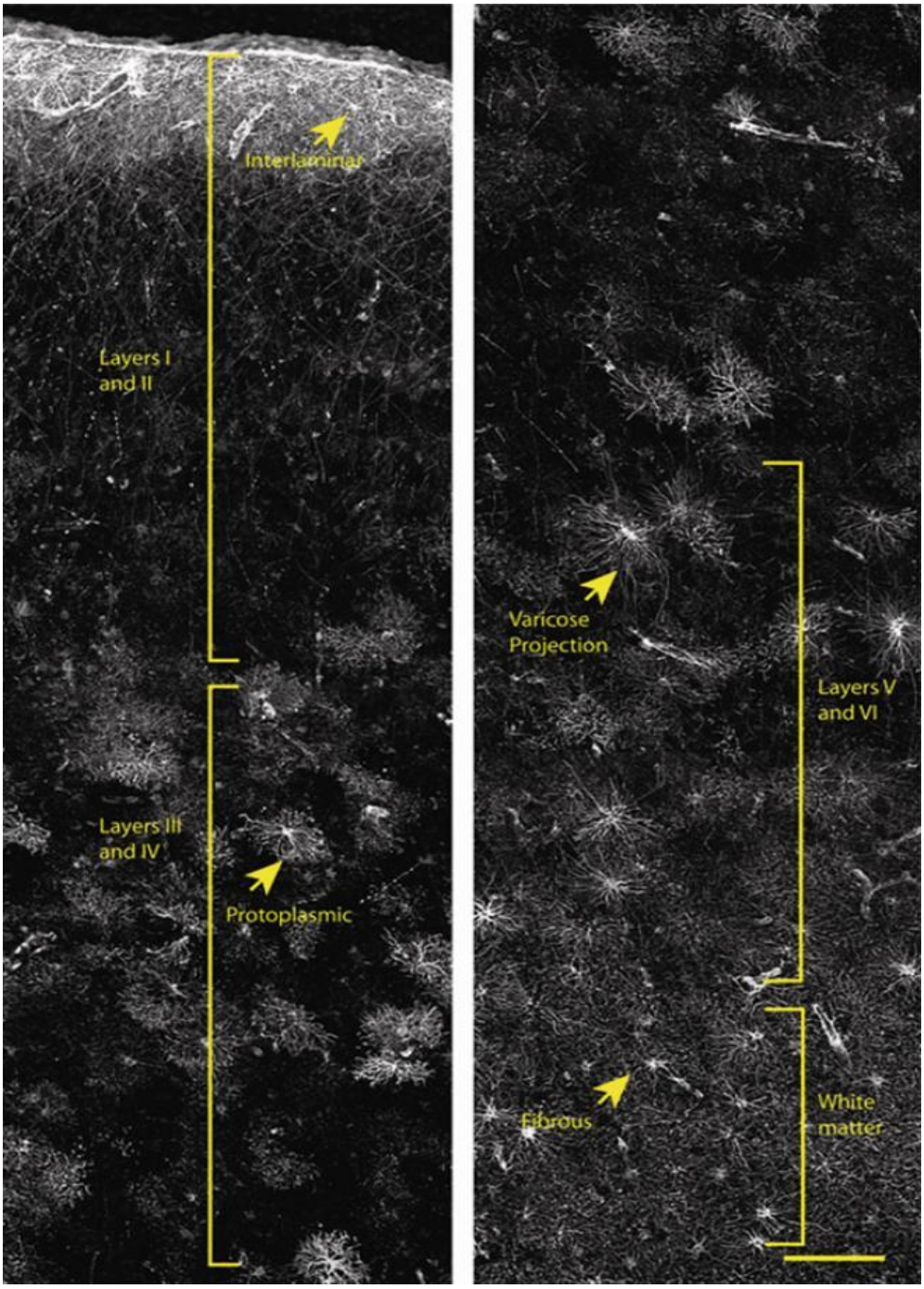

Figure 2.1 Four major types of astrocytes cells. Layer I is composed of the cell bodies of interlaminar astrocytes, whose processes extend over millimetre lengths through layers II–IV and are characterized by their tortuous morphology. The most common type of astrocytes, Protoplasmic astrocytes reside in layers II–VI. Varicose projections astrocytes are found only in humans and are seen sparsely in layers V–VI. Fibrous astrocytes are found in the white matter and contain numerous overlapping processes. *Yellow lines* indicate areas in which the different classes of astrocytes reside. Scale = 150 μm (Oberheim et al, 2012).

2.6 Functions of astrocytes

2.6.1 The brain micro-architecture

In mammalian brain, astrocytes play a role as a definer for the parenchyma specifically in its micro-architecture structure. Through the process “tiling”, it divided the grey matter into relatively independent structural units. The protoplasmic type of astrocytes engages their own position and constructs the micro-anatomical domains within the limits of their processes. The membrane of the astrocyte within these domains, not just covers synapses and neuronal membranes, but also sends processes to coat the wall of neighbouring blood vessel with their endfeet. This kind of complex form consists of astrocyte-neuron-blood vessel is known as neurovascular unit (Freeman, 2010; Jakobs, 2014; Muoio et al, 2014).

2.6.2 Extracellular homeostasis in brain

Astrocytes also play a duty as one of the gate keepers in brain as it controls concentrations of ions, neurotransmitters and metabolites and also regulate the movement of water. It also induces and ensures the stabilization of neuronal synapses (Clarke and Barres, 2013). One of the recognized functions of astrocytes in order to maintain the homeostasis in brain is controlling the level of K^+ concentration. The K^+ concentration arise from its resting state as there are present of neuronal activity. The accumulation of K^+ concentration level in extracellular space modulates may initiate epileptic seizures (Florence et al, 2012; Molofsky et al, 2012).

There are two common mechanisms that were used by astrocytes to remove this excess extracellular K^+ (Scemes and Spray, 2012). One of the mechanisms is a passive mechanism known as 'spatial buffering'. The mechanism work by redistributed within the astrocyte or the coupled astrocytes network after reuptake the K^+ at the higher concentration and then released at sites with lower concentration. Besides that, astrocytes can discard the excess K^+ by increase the pump activities. As the Na^+/K^+-ATPase activity increases, the intracellular K^+ will be increased. The glial syncytia and aquaporine channels expressed in astrocytes also play a role in water homeostasis in the brain (Scemes and Spray, 2012; Hertz et al, 2013).

2.6.3 Removing of Glutamate

Glutamate, major excitatory neurotransmitter in brain will act as a powerful neurotoxin when it releases more than required amounts or being excess for a long time. This neurotoxin may trigger neuronal cell death in numerous acute or chronic brain lesions. Function of astrocyte in taking up transmitter was first described by Ernesto Lugaro, an Italian psychiatrist in 1907 (Kettenmann and Verkhratsky, 2008). Astrocytes remove a huge amount of glutamate from extracellular space, about 80% of the glutamate released while neurons take the remaining 20%. Astrocyte cells remove the glutamate through excitatory amino acid transporters (EAAT) which present in five types in human brain but two expressed exclusively in astrocyte; EAAT1 and EAAT2 (Hayashi and Yasui 2015; Kinoshita et al 2016).