Changes of GABA Receptor and Immunoreactivity Modification of IP_3 & BDNF-TrkB in KA-Induced Rat Epileptogenic Hippocampal Neuronal Culture

BY

CHONG PEI NEI

Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Neuroscience

FEBRUARY 2018

ACKNOWLEDGEMENT

I would first like to thank my thesis supervisor, Dr Mohammed Faruque Reza, for being an extremely supportive and kind supervisor throughout my research progress. He provided me plenty of independence in my research, whilst never failing to make sure that I was on the right track. I would also like to thank my co-supervisor, Dr Muthuraju Sangu, for being very helpful and generous with guidance. In addition, I would also like to thank Tee Jong Huat who provided me with much guidance with laboratory works, techniques and data collection at the beginning of my study. I am grateful to have them for their critical comments and advice, without which my research would not be as smooth. I would also like to thank Department of Neuroscience and School of Medical Science which providing the facilities to allow my research run smoothly. I would also like to offer my sincere gratitude to my fellow coursemates and colleagues in the Department of Neuroscience who assisted me throughout my research and data collection. I would also like to extend my gratitude to Bio-Diagnostic Sdn. Bhd and their staffs to provide me the facilities (EVOS FL Auto 2) which completed cell viewing and image capturing for immunocytochemistry. Finally, I must thank my family and friends for their support and encouragement throughout my degree.

This research was supported by Fundamental Research Grant Scheme (FRGS): 203/PPSP/6171175

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Akt	: serine/threonine kinase Akt (also known as protein kinase B (PKB)
BDNF	: Brain derived neurotrophic factor
CREB	: cAMP response element-binding protein
DAG	: Diacylglycerol
DAPI	: 4',6-diamidino-2-phenylindole
DIV	: Day *in vitro*
DPBS	: Dulbecco's Phosphate-Buffered Saline
E-18	: Embryonic day 18
ERK	: Extracellular signal-regulated kinases
GABA	: γ-Amino butyric acid
$GABA_AR$	: γ-Amino butyric acid A receptor
$GABA_A$ α1-containing R	: γ-Amino butyric acid A alpha-1 subunit containing Receptor
GAT	: GABA transporter
GLUR5	: Glutamate receptor, ionotropic, kainate 1, also known as GRIK1
HBSS	: Hank's Balanced Salt Solution

ILAE : International League Against Epilepsy

IP_3 : Inositol 1, 4, 5 triphosphates

IP_3R : Inositol 1, 4, 5 triphosphate receptor

KA : Kainic-acid

KCC2 : Potassium-chloride transporter member 5

MAPK : Mitogen activated protein kinases

MAP2 : Microtubule-associated protein 2

mRNA : Messenger RNA

NMDAR : N-methyl-D-aspartate receptor

NR1 : N-methyl-D-aspartate receptor subunit NR1

NR2A : N-methyl-D-aspartate receptor subunit NR2A

pro-BDNF : pro-brain derived neurotrophic factor

$p75^{NTR}$: neurotrophin receptor p75(NTR)

PBS : Phosphate buffer saline

PFA : Paraformaldehyde

PLC : Phopholipase C

PLCγ : Phopholipase Cγ

PSD-95 : Postsynaptic density protein 95

PI3K : Phosphatidylinositol-4,5-bisphosphate 3-kinase

TrkB : Tyrosine kinase receptor B

ROS : Reactive oxygen species

Cl^- : Chloride ion

Ca^{2+} : Calcium ion

Na^+ : Sodium ion

NO : Nitric Oxide

K^+ : Potassium ion

NaH_2PO_4 : Sodium Phosphate Monobasic

Na_2HPO_4 : Sodium Phosphate Dibasic

NaCl : Sodium Chloride

LIST OF SYMBOLS

α	: Alpha value; Significance level
α1	: Alpha-1
α2	: Alpha-2
α3	: Alpha-3
α4	: Alpha-4
α5	: Alpha-5
α6	: Alpha-6
β	: Beta
β2	: Beta-2
β3	: Beta-3
CI	: Confidence interval
δ	: Delta
F	: F-statistic
γ	: Gamma
γ2	: Gamma-2
wt	: molecular weight
p	: *p*-value

ρ	: Rho
SD	: Standard deviation
V	: Volume

ABSTRAK

PERUBAHAN RESEPTOR GABA DAN MODIFIKASI IMMUNOREAKTIVITI IP_3 & BDNF-TrkB DALAM KULTUR NEURON EPILEPTOGENIK HIPPOCAMPAL TIKUS YANG TERINDUKSI OLEH KAINATE ASID

PENGENALAN: Penyakit epilepsi adalah keadaan biasa yang berlaku dalam kira-kira 50 juta orang di seluruh dunia. Serangan epilepsi adalah keadaan sementara yang berlaku akibat daripada aktiviti berlebihan yang tidak normal di dalam otak. Kajian menunjukkan reseptor $GABA_AR$ menyokong peranan BDNF-TrkB sebagai faktor pelindung neuron dalam *hippocampus* dan BDNF-TrkB yang mencetuskan salah satu isyarat IP_3R yang terlibat dalam serangan epilepsi. Kajian terdahulu memberi tumpuan kepada Teknik *in vivo* dan Teknik pengkulturan secara *in vitro*. Walau bagaimanapun, kajian semasa menunjukkan terdapat hubung kait yang tidak jelas, oleh itu keputusan daripada kajian semasa boleh dilaksanakan melalui pengkulturan neuron *hippocampal* tikus yang diekstrak secara *in vitro.*

OBJEKTIF: Kajian ini bertujuan untuk mengkaji perubahan reseptor $GABA_A$ ($GABA_AR$), faktor neurotropik otak (BDNF), reseptor kinase Tyrosine B (TrkB) dan reseptor Inositol 1, 4, 5 trifosfat (IP_3R) dalam neuron hippocampal normal dan epileptogenic disebabkan oleh asid kainik.

METODOLOGI: Dalam kajian ini, kaedah pencetus sawan secara kimia, asid kainik (KA) telah digunakan untuk melibatkan kesan sawan di dalam neuron hippocampus tikus. Embrio hari ke-18 (E-18). Sel neuron daripada tikus E-18 telah dikultur berdasarkan kaedah yang telah dioptimumkan dengan menggunakan kepingan kaca bersalut *poly-L-lysine* (Todd et al., 2013). Pada hari ke- 14 dengan mengunakan teknik *in vitro* sel kultur neuron akan dirawat dengan 0.5µM KA selama 30 (KA30), 60 (KA60) dan 90 (KA) minit. Neuron yang normal dan neuron yang dirawat dengan KA masing-masing akan menjalani pemeriksaan bagi daya tahan sel, pertubuhan neurit dan penilaian immune kimia.

KEPUTUSAN: Hasil kajian menunjukkan receptor $GABA_A$ mengandungi komponen α1 dan BDNF yang dirawat KA adalah tidak signifikan berbanding pengkulturan sel neuron yang dirawat secara normal. Reseptor TrkB yang diberi rawatan menunjukkan kadar penurunan yang signifikan ($F (3, 8) = 8.761$, $p < 0.01$). Pada KA30, KA60 dan KA 90 menujukkan penurunan yang signifikan jika dibandingkan dengan kumpulan kawalan iaitu kumpulan yang tidak dirawat dengan KA. Selepas rawatan mengunakan KA, IP_3R juga meningkat dengan ketara jika dibandingkan dengan kumpulan kawalan ($F (3, 8) = 6.954$, $p < 0.05$).

KESIMPULAN: Rawatan KA dalam pengkulturan sel neuron hippocampal menunjukkan peningkatan ketara dalam ekspresi reseptor TrkB (reseptor BDNF) dan IP_3R, manakala imunoreaktiviti BDNF dan reseptor $GABA_AR$ yang mengandungi

komponen α1 menunjukkan kadar penurunan yang tidak signifikan. Secara keseluruhannya, kajian ini menunjukkan bahawa rawatan mengunakan KA ke atas sel neuron hippocampal tikus secara *in vitro* mempengaruhi *epileptogenesis* dan melibatkan perubahan dalam ekspresi reseptor $GABA_A$ mengandungi komponen α1, BDNF, TrkB dan IP_3R.

Kata Kunci: GABA, $GABA_AR$, $GABA_A$ R yang mengandungi α1, BDNF TrkB, IP_3R, Kainic asid, Epileptogenik.

ABSTRACT

Changes of GABA Receptor and Immunoreactivity Modification of IP_3 & BDNF-TrkB in KA-Induced Rat Epileptogenic Hippocampal Neuronal Culture

INTRODUCTION: Epilepsy seizure is a common condition occurring in approximately 50 million people worldwide. Epilepsy seizure is a transient occurrence of sign and or symptoms due to abnormal excessive or hypersynchronous activity in the brain. Studies showed $GABA_AR$ supporting the role of BDNF-TrkB as a neuroprotective factor in the hippocampus and BDNF-TrkB triggers one of the downstream signalling IP_3R which is involved in epilepsy seizure. However, past studies focus on *in vivo* and *in vitro* organotypic hippocampal slices culture. It is unclear whether the findings of the current study would extrapolate *in vitro* dissociated primary rat hippocampal neuron culture.

OBJECTIVE: To study the changes of γ-Amino butyric acid A receptor ($GABA_AR$), Brain derived neurotrophic factor (BDNF), Tyrosine kinase receptor B (TrkB) and Inositol 1, 4, 5 triphosphate receptor (IP_3R) in normal and epileptogenic hippocampal neuron.

METHOD: In this study, chemo-convulsant method, kainic acid (KA) induced seizure applied to (E-18) rat hippocampus neuron. The E-18 rat hippocampus neuron

was cultured based on the optimised method by using poly-L-lysine coated glass coverslips (Todd *et al*., 2013). The day *in vitro* (DIV), 14 culture neurons induced with 0.5μMKA for 30 (KA30), 60 (KA60) and 90 (KA)minutes respectively. The normal and KA treated neuron underwent cell viability, neurite outgrowth density assessment and immunocytochemistry assessment respectively.

RESULTS: Finding showed no significant effects of $GABA_A$ α1-containing receptors ($GABA_A$ α1 containing R)and BDNF of the KA treatment to the normal neuron culture. The TrkB receptor was significantly decreased by the treatment ($F (3, 8) = 8.761$, $p < 0.01$). The KA30, KA60 and KA 90 were significant decreased compared to the control group with no KA treated culture. The IP_3R was elevated significantly y the treatment of KA ($F (3, 8) = 6.954$, $p < 0.05$) .

CONCLUSIONS: The KA administration on hippocampal neuron culture showed an increase expression in the TrkB (BDNF receptor) and IP_3R significantly whereas, the immunoreactivity of BDNF and $GABA_A$ α1 containing R showed a decreased expression that is not statistically significant. Altogether, our study showed that KA administration on *in vitro* rat hippocampal neuron induces epileptogenesis which, results in changes in the $GABA_A$ α1 containing R, BDNF, TrkB and IP_3R.

Keyword: GABA, $GABA_AR$, $GABA_A$ α1 containing R, BDNF, TrkB, IP_3R, Kainic acid

CHAPTER 1

INTRODUCTION

1.0 Epilepsy and seizure

According to International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE), epileptic seizure is a transient occurrence of sign and or symptoms due to abnormal excessive or hypersynchronous neuronal activity in the brain. Meanwhile, epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizure and by the neurobiological cognitive, psychological and social consequences of this condition. According to Fisher (2005), to be defined as epilepsy it requires the occurrence of at least one epileptic seizure. Currently, statistic shows approximately 50 million people live with epilepsy worldwide, with the estimation 2.4 million new patients are diagnosed with epilepsy every year (World Health Organization, 2017). Considering the total world population, less than 1% having epilepsy and about one-third of patients are detected with refractory epilepsy where the seizures are not controlled by two or more appropriate antiepileptic medications or other therapies (Stafstrom & Carmant, 2015).

Although the term of epilepsy and seizure are often to be misunderstood, seizure is difference than epilepsy. Seizures can be divided into three categories; generalised, focal and epileptic spasm according to ILAE most recent publication on classification of epileptic seizures and epilepsies (epilepsy syndrome) in 2010. Generalised epileptic

seizures are conceptualised as a seizure originating at some point within and bilateral distributed network (Berg *et al*., 2010). An individual seizure onset can be localised which the location and lateralization are inconsistent from one seizure to another. Whereas, focal epilepsy seizures are conceptualised as a seizure originating within network limited inside one hemisphere and the site of onset is consistent (Berg *et al*., 2010). Focal epilepsy seizures are depending on the cortex involved, for instance, seizure onset at temporal lobe are often results dyscognitive (Stafstrom & Carmant, 2015). Dyscognitive is a condition when consciousness is impaired during focal seizure, patient unable to responds verbal or tactile stimuli and exhibit uncoordinated activity such as paraesthesia (Stafstrom & Carmant, 2015). The third category of seizure is epileptic spasm, which is relatively uncertain. Epileptic spasms are manifested by sudden extension or flexion of extremities, held for several seconds, and they recur in clusters for example, sudden extension of both arms and flexion of the neck (Berg & Millichap, 2013). These epilepsy syndromes are detrimental, meaning it requires continuous explorations to find a resolution to relieve epilepsy's patient. Numerous of researches have been conducted on animal to study the onset of seizures in human and understanding the mechanism underlying seizure.

1.1 Animal model in epilepsy studies

Among the approaches are different types of animal model, which have been used in studies relating to epilepsy for decades. Animal model is essential in elucidate mechanism underlying epilepsy seizures with the aims to mimic the mechanism of epilepsy in human. Generally, animal models are used in epilepsy studies involving of epileptic seizures rather than model of epilepsy considering the fact that epilepsy is

characterized by occurrence of epilepsy seizure more than one time, in which an acute seizure is electrically induced in normal non-epileptic animal unable to represent a model of epilepsy (Löscher, 2011). Other than that, there are more genuine models of epilepsy such as genetic animal model, which are mutated or transgenic with spontaneously recurrent seizures exhibiting closely similar seizure symptoms in human epilepsy (Löscher, 2011). On the other hand, induction of seizures in normal animals or commonly known as epileptogenesis can be induced by chemical substances such as pilorcarpine and kainic acid (KA) as well as electrical or kindling technique (Löscher, 2011). Epileptogenesis refers to a process in which the occurrences of spontaneous seizure induced by brain-damaging insults that triggers a cascade of molecular and cellular changes (Pitkänen, & Lukasiuk, 2009). Chemical induced seizure is known as chemoconvulsant while electrical induced seizure is referred as electrical stimulation. Both chemoconvulsant and electrical stimulation animal models are used specifically based on the experimental method.

Pilocarpine and KA are chemoconvulsants and have been used to generate spontaneous recurrent seizures. Pilocarpine is a muscarinic acetylchlorine receptor agonist, which is usually used to induce seizure in limbic via systemic or intracerebral injection of pilocarpine, whereas KA is a glutamate analogue and its causes neuronal depolarization and seizures preferentially targeting the hippocampus through systemic or intracerebral administration (Kandratavicius *et al*., 2014). Both pilocarpine and KA produce neuronal damage without sophisticated instruments. Pilocarpine and KA are exhibited similar electroencephalographic features and the neuropathological alteration in rodents (Lévesque, Avoli & Bernard, 2016). However, pilocarpine model gives more intense damage than KA model and KA is favorable to be used as initial screening of

neuroprotective drug (Covolan and Mello, 2000). Pilocarpine-induced seizure is often used to examine the relationship of between mossy fiber synaptic reorganization a spontaneous seizure activity whereas KA-induced seizure is targeting hippocampus (Kandratavicius *et al*., 2014; Reddy & Kuruba, 2013). A major drawback of the KA is the KA model is variable sensitivity of rat of different strains, sex, age and weight to KA where aged rat is more vulnerable to KA with high potency towards KA with greater neuronal damage (Reddy & Kuruba, 2013). Another limitation is KA has direct excitotoxic action making it difficult to differentiate direct neuronal damage from seizure-induced neuronal damage and it is less ideal for anticonvulsant for SE because not all effective drugs are truly effective in KA-induced model (Reddy & Kuruba, 2013).

If compared to KA-induced model, kindling is the most studied model of electrical stimulation. In this method, the emergence of spontaneous seizure and the establishment of permanent epileptic states are culminated through electrical stimulation in a specific brain region (Kandratavicius *et al*., 2014). This model has advantages in reproducing epileptogenic features in the intact brain with low mortality and high reproducibility (Kandratavicius *et al*., 2014). However, seizure modeling by electrical stimulation does not provide cell-type specific in the brain and this stimulation protocols could be costly and laborious when used for chronic studies (Kandratavicius *et al*., 2014).

In *in vitro* perspective, KA-induced model is more appropriate to be used. KA-induced seizures preferentially targeting hippocampus and its effect onto seizure is less intense compared to pilocarpine this allows our preliminary study on *in vitro* model from missing potential components. Moreover, KA has validated effect from previous studies and this perhaps contribute a better understanding of the molecular, cellular and

pharmacological mechanism underlying epileptogenesis and ictogenesis (Lévesque, & Avoli, 2013). Therefore, KA-induced seizure model is preferentially to be used in neuron culture.

1.1.1 KA-induced model

KA-induced model or KA model is a tool for seizure studies due to manifestation of symptoms such as behavioral changes of rodents, oxidative stress, glial activation, and production of inflammatory mediators, endoplasmic reticulum stress, mitochondrial dysfunction and neuronal degeneration in the brain of rodents (Nadler, 1981; Robinson & Deadwyler, 1981; Sairazi *et al*., 2015). KA is a L-glutamate and an agonist of ionotropic KA receptors (Lévesque and Avoli, 2016). KA was isolated and extracted in the early 1950s from red algae (*Digenea simplex*). KA model is practically used to induce epileptogenic symptoms in experimental animal model. Experimental models that administrated with KA exhibit seizure, behavioral changes, oxidative stress, and production of inflammatory mediator (Sairazi *et al*., 2016). KA is commonly used as a model of epilepsy either in neuronal culture model or *in vivo* model (Lévesque & Avoli, 2016; Pal *et al*., 2001). KA essential in experimental models and it was found that occurrences of epileptogenesis is due to the alteration of Ca^{2+} homeostatic mechanism (Sun, 2001). Alteration of Ca^{2+} happened when KA bound to the kainate receptors excessively and over-activation of kainite receptor produces neuronal membrane depolarization causes excessive influx of Ca^{2+} into the neurons which is called glutamatergic excitotoxicity (Bevinahal *et al*., 2014; Halopainen, 2004). Eventually, excessive influx of Ca^{2+}contributes to neuronal insult and neuronal death (Bevinahal *et*

al., 2014; Halopainen, 2004, Sun *et al*., 2001; Wang *et al*., 2006; Zheng *et al*., 2010). Therefore, dose of KA application to the neuron is critical as the effects of KA-induced seizure are detrimental.

Dosage of KA application is a critical factor in this model as the different dosage would results in varies of severity of seizure symptoms in the experimental model. Low dosage of KA is always recommended. As in, the conduction of screening for the dosage and time of KA exposure are certainly needed to be considered to study the changes in hippocampal neuron culture. Based on Järvelä (2011) experiment, immature (P6, P9, Wistar and Sprague Dawley rat) and mature juvenile (21-day old Sprague Dawley rat) organotypic hippocampal brain slices were administrated with three different concentrations of KA, which are at concentrations of 1, 2, and 5 μM. Results demonstrated 1 μM was generally mild; 2 μM is mild to moderate and 5 μM is moderate to severe to organotypic hippocampal brain slices. These suggested that lower doses of KA should be used to control the mortality issue in immature rats (Järvelä (2011). Kaneko and colleagues (2015) also used KA to induced early-stage epilepsy in *in vitro*. A range of 1.25 nM to 125 μM KA was administrated onto day-3 cultures primary neuronal cells for 3 days. The half maximal inhibitory concentration (IC_{50}) was 5 μM KA, where the KA was found effectively inhibiting seizures function in the primary neuron culture (Kaneko *et al*., 2015). Therefore, KA dose is varying to different experimental approach to exhibit seizure.

1.2 Epilepsy and the responsible receptors

Previous works in epilepsy studies have demonstrated that ionic changes in the excitatory/inhibitory balance of the brain play a crucial part in epilepsy (Kim & Yoon, 2017). For example, gamma-aminobutyric acid or commonly known as GABA is the principle inhibitory neurotransmitter in the cerebral cortex which maintaining the inhibitory tone that counterbalances neuronal excitation by binding on $GABA_A$ receptors ($GABA_ARs$). When this balance is disturbed, seizures which followed by epilepsy may occur. In addition to GABA and $GABA_ARs$ interactions, brain-derived neurotrophic factor (BDNF) and its receptor tyrosine kinases (TrkB), which are essential in influencing neuronal survival, differentiation, synaptogenesis and maintenance (Webster *et al*., 2006), also involve in the limbic epilepsy animal model (Danzer, 2004). Mice with neuron-specific TrkB deficient and transgenic mice with overexpressing BDNF demonstrated that TrkB activation is critical for downstream signaling for epileptogenesis (Croll *et al*., 1999; He *et al*., 2004). A series of evidence also shows inositol (1,4,5)-trisphosphate receptors or IP_3R in hippocampus is important in regulating the intracellular Ca^{2+} as in modulating anticonvulsant and epileptogenic effect and in mediating injury during seizures (Nagarkatti *et al*., 2008). In transgenic mice with deficient of phenotype of IP_3R in cerebellum and brain stem exhibited cerebellar ataxia and seizure like posture at postnatal 10 days and this mouse have short life span it die within 3 - 4 weeks after birth (Hitsatsune & Mikoshiba, 2017).

1.2.1 GABAergic mechanisms

$GABA_AR$ which is the main inhibitory receptor is one of the ion channels that had been widely used to study epilepsy. Generally, $GABA_AR$ is made up of five subunits to form a pentameric structure with the chloride channel in the middle of the complex. The subunits are group into different group which comprising 19 members; α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3 (D'Hulst & Kooy , 2007; Tsunashima *et al*., 1997). $GABA_AR$ can be activated by GABA which rapidly removed by uptake into both glia and presynaptic nerve terminal and then catabolized by GABA transaminase that enable the neuron to counterbalance the flows of ions rapidly during the neuronal excitation (Kim & Yoon, 2017).

The various combinations of different subunits produce specific traits that are localized to different brain regions with different pharmacological properties (Olsen & Sieghart, 2009). In the body, $GABA_ARs$ present in the cortex, heart, lung and stomach, however they can be found relatively higher in the certain places such as renal medulla, cortex, left ventricle, aorta and pancreas. The mRNA of $GABA_AR$ in the cortex was found different from mRNA of $GABA_AR$ at other organs of the body, researcher also found out that $GABA_AR$ subunit α1, β1, β3 and γ3 are exclusively present in the brain only (D'Hulst & Kooy, 2007). Therefore, $GABA_AR$ is one of the main receptor in neuronal study to elicit the functional, morphology as well as the sites for therapeutic agent (D'Hulst & Kooy, 2007; Kim & Yoon, 2017; Nishimura *et al*., 2005; Tyagi *et al*., 2007).

At early stage of human development, $GABA_AR$ exhibited as excitatory but later it transforms to inhibitory at the point of maturity as discussed by Ben-Ari (2006) in his review stated that $GABA_AR$ is excitatory at early developmental stages, low density of glutamatergic synapse is insufficient to result, a high-frequency oscillation that has long-term consequence without the GABA act as a paracrine factor. Moreover, researchers also found $GABA_AR$ functions as an excitatory neurotransmitter-triggers membrane depolarization, action potential and open Ca^{2+} channel plasma membrane at early of neuronal development (Obrietan, Gao & van den Pol, 2002).

In mature brain, $GABA_AR$ activation leads to Cl^- influx, and results hyperpolarization in the neuron. According to Senargor, (2010) tonic GABA binds to low affinity extra-synaptic $GABA_AR$. This is due to the low expression of KCC2 pump and high expression of the NKCC1 pump. The KCC2 function is controlling the trafficking of cation-Cl^- and the KCC2 shift the GABAergic receptor from excitation to inhibitor as maturation progress (Blaesse *et al*., 2009). The GABAergic transition from excitation to inhibition is activity-dependent. An initial *in vitro* study reported that GABAergic activity regulates its own switch from excitation to inhibition (Ganguly *et al*., 2001). Along the maturation of KCC2, low threshold T-type Ca^{2+} channels are opened, resulting in Ca^{2+} influx and intracellular events that will eventually lead to neurite extension. At late maturational stages, glutamatergic signaling has developed and GABA may act in together with NMDA and AMPA neurotransmission, resulting in stronger depolarization and the opening of high-threshold L-type Ca^{2+} channels. This will result in stronger Ca^{2+} influx, which may be important for triggering downstream intracellular events that are responsible for the late phases of neurite growth (Sernargor,

2010). This process will come to an end when synaptic inhibition mature through changes in the composition of the $GABA_AR$ subunits and reversal of the Cl^- gradient, leaving the possibility for plastic remodeling of the existing neurite network by the dynamic balance of neuronal activity resulting from hyperpolarizing GABA and fully developed glutamatergic signaling.

1.2.2 BDNF

Previous studies showed that brain derived neurotrophic factor or BDNF interacts with GABA transport as well as $GABA_ARs$ in nerve terminals and neuronal cultures. For instance, Obrietan and colleagues (2002) demonstrated that $GABA_AR$ activation triggered BDNF expression and pre-synaptically facilitated GABA release thus forming a positive feedback loop during the maturing of BDNF at early development, where GABA stimulates BDNF expression and BDNF facilitates the synaptic release of GABA (Obrietan *et al*, 2002). MAPK cascade and transcription factor CREB should be coupled and they reviewed NT3 exerted an excitatory modulation of GABA activity, but, only during the development period when GABA function as an excitatory neurotransmitter (Liu *et al*., 1999). Jagasia and the team (2009) showed GABA-mediated depolarization was also involved in the phosphorylation of CREB during the first 2 weeks of development. The BDNF was then envisaged CREB signaling may eventually converge onto the BDNF pathway and act as an effector of depolarizing GABA effect on neurite outgrowth and dendritic length (Chan *et al*., 2008). In different work by Sernagor and colleagues (2010), they deduced that at early development of neuron the release of BDNF are depends on GABAergic depolarization,

however the synthesis of BDNF mRNA was found to reduce in response to GABAergic activity in the mature neuron (Sernagor *et al*., 2010). Deprivation of BDNF signaling results in dendrite growth impairment in newborn neurons during development and eventually converge onto BDNF pathway for the dendritogenesis effect, due to the postnatal depletion of BDNF was impactful to dendrite branching, length and complexity (Berghuis *et al*., 2006; Giusi *et al*., 2009; Sernagor *et al*.,2010).

BDNF is ubiquitous in central nervous system and relatively high in the hippocampal formation and cerebral cortex. BDNF is a part of a general mechanism for activity-dependent modification of synapses in developing and adult nervous system. In rats, BDNF level is expressed highly in the early and the postnatal period and throughout the life of rodent (Sakharnova, 2012; Webster *et al*., 2006). Gonzalez (2014) reviewed that BDNF is at the highest level in the hippocampus, where its concentration is increased approximately 20 times higher during the first week of young adulthood. This is revealed the level of neurotrophic is growing rapidly in the early development of hippocampus. BDNF increases the survival and promoting the growth of dendrite in the brain.

BDNF is a member of 'neurotrophin' family of the neurotrophic factor. The amino acid sequence of BDNF have a strong homology with nerve growth factor, neurotrophin first described due to its trophic (survival and growth promoting) effects on the sensory and sympathetic system such as neurotrophin-3 and neurotrophin-4/5 (Binder, 2004). BDNF expression also can be promoted by the exercise where, Sleiman and his colleagues (2016) concluded that BDNF, a trophic factor that supplementary to cognitive improvement and relieve depression and anxiety. Besides, BDNF also being

studied in peripheral taste (Huang & Krimm, 2010) and BDNF contributed in mechanical nociception production after nerve injury in the spinal cord (Wang *et al*., 2009).

1.2.3. BDNF and TrkB

Along with its receptor; tyrosine kinases (TrkB), BDNF influence neuronal survival, differentiation, synaptogenesis and maintenance (Webster, Herman, Kleinman & Weickert, 2006). BDNF-mediated TrkB activation was involved in the limbic epilepsy animal model (Danzer, 2004). The TrkB activation would trigger three downstream signaling pathways; Phosphatidyl inositol3-kinase (PI3K)- , Phospholipase Cγ (PLCγ) and Mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) (Tanaka *et al*., 1997; Matthew & Hablitz, 2008; Yoshii & Constantine-Paton, 2014). Yoshii and Constantine-Paton (2014) found out that TrkB regulates postsynaptic localization of PSD-95 through three downstream pathways, MAPK/ERK can regulate transcription through CREB and other transcription factors whereas MAPK/ERK and PI3K-AKT pathway play a major role in translation. The post-translational modification is regulated by BDNF-TrkB signaling via PLC-PKC. These mechanisms are likely to regulate BDNF-dependent long-term plasticity.

Yang and colleagues (2016) suggested PLCγ is the pathway involved in epileptogenic seizure. Additionally, this PLCγ pathway generates inositol triphosphate (IP_3) thru activation by TrkB, which mobilizes intracellular Ca^{2+} from the endoplasmic

reticulum (Tanaka *et al*., 1997; Krishnakumar, 2009; Yang *et al*., 2016). The mobilization of Ca^{2+} ions are eventually result in suppression of $GABA_AR$ and leads to epileptogenic seizure (Heinrish, 2011; Mathew & Hablitz, 2008).

1.2.4 IP_3R and epilepsy

IP_3R was found higher in epileptic cerebral cortex compare to normal cortex (Krishnakumar, 2009). The IP_3R are the intracellular Ca^{2+} release channels that play a key role in Ca^{2+} signaling in cells localized predominately in the endoplasmic reticulum of all cell type (Bezprozvanny *et al*., 2005; Foskett *et al*., 2007). IP3 are commonly known to regulate membrane trafficking, glucose metabolism and signal transduction (Yang *et al*, 2016). It plays a significant role in postsynaptic transmission, such as synaptic transmission. The IP_3R is a secondary messenger molecule for release Ca^{2+} from intracellular stores when IP_3 is bound to the endoplasmic reticulum (Hitsatsune & Mikoshiba, 2017). According to Nagarkatti and colleagues (2008), IP_3R in hippocampus is important in regulating the intracellular Ca^{2+} as in modulating anticonvulsant and epileptogenic effect and in mediating injury during seizures. In transgenic mice with deficient of phenotype of IP_3R in cerebellum and brain stem exhibited cerebellar ataxia and seizure like posture at postnatal 10 days and this mouse have short life span it die within 3 - 4 weeks after birth (Hitsatsune & Mikoshiba, 2017). Thus, the IP_3R is essential to modulate the cell signaling and cell survival.

1.3 Rational of the study

Previous works show BDNF's mRNA expression increased after KA administration (Bevinahal *et al*., 2014; Ernfors *et al*., 1994; Isackson *et al*., 1991; Kokaia *et al*., 1995). In addition to that, a study conducted by Lähteinen and colleagues (2002) on BDNF transgenic mice with truncated TrkB receptor demonstrated that seizure behavior was reduced in response to KA. Also, Koyama and the team (2005) found out that activity-induced BDNF release and Trk receptor activity protected the hippocampus from developing of hyper-excitable circuit by activating $GABA_AR$ supporting the role of BDNF as neuroprotective factor in the hippocampus. Therefore, it is important to examine the changes of $GABA_AR$, BDNF, TrkB and IP_3R in normal and KA-induced epileptogenic neuron to elucidate the mechanism of epileptogenesis and apparently relieves patients who suffered from seizure by provide therapeutic target for anti-epileptic drug research.

1.4 Objective and hypothesis

1.4.1 Hypothesis.

Changes of $GABA_A$ receptor and immunoreactivity properties of BDNF, TrkB receptor and IP_3R may occur in the rat epileptogenic hippocampal neuron culture.

1.4.2 Research objective

1.4.2.1 General objective

To study the changes of $GABA_A\alpha1$ containing Receptor, BDNF, TrkB and IP_3R in the normal and KA-induced rat epileptogenic hippocampal neuron culture.

1.4.2.2 Specific objective

1. To optimize time of KA administration on E-18 Rat hippocampus neuronal culture.
2. To assess the expression of immunoreactivity $GABA_A\alpha1$ containing Receptor, BDNF, TrkB and IP3R in normal and epileptogenic hippocampal neuron treated with KA.

CHAPTER 2

MATERIALS AND METHODS

2.0 Materials

2.0.1 Primary Neuron culture

Primary Rat Hippocampal Neuron (C/N: A104841-01) was purchased from Gibco™, USA. Primary Rat Hippocampus Neurons were isolated from day-18 Fisher 344 rat embryos (E-18) and cryopreserved in a medium containing DMSO. The neurons were stored and transported in a cryovial at -196 °C dry liquid nitrogen tank.

2.0.1.1 Culture media

The neurons cultured using Neurobasal®-medium, GlutaMAX™- I Supplement and B-27® supplement was prepared according to the protocol provided by Gibco™. The Gibco™ Antibiotic-Antimycotic was used acts as antibiotic for throughout the cultures. L-glutamine was supplemented in the first 4 days of culturing as amino acid supplement for neuron culture media. The composition of Neurobasal complete media is showed in Table 2.1.

Table 2.1 The composition of Neurobasal complete media in 100 mL.

Component	**Final concentration**	**Volume (mL)**
Neurobasal®-medium	1x	98
GlutaMAX™- I Supplement	2 %	2
B-27® supplement	0.5 mM	0.25
L-glutamine *	250 µM	0.0625
Antibiotic-Antimycotic	0.01 %	0.5

*Only supplemented at the first 4 day of culture

2.0.2 Chemicals and reagents

Chemicals and reagents used in the experiment were listed below in Table 2.2.

Table 2.2 List of chemicals and reagents in the study.

Materials	**Manufacturer**	**Application**
Ethanol denatured	HmbG, Germany	Cell culture
2-propanaol, ACS Grade for analysis	Merck Millipore, USA	
Poly-L-Lysine (PLL)	Sigmal-Aldrich®, USA	
Paraformaldehyde (PFA)	Merck Millipore, USA	Immunostaining
Sodium Chloride, NaCl	Riedel-de Haen, Denmark	
Sodium Phosphate Monobasic, NaH_2PO_4	Sigmal-Aldrich®, USA	
Sodium Phosphate Dibasic, Na_2HPO_4	Sigmal-Aldrich®, USA	
Sodium Hydroxide anhydrous, NaOH	Sigmal-Aldrich®, USA	Immunostaining
Triton™ X-100 Surfact-Amps™ Detergent Solution	Thermo Scientific, USA	
Goat-serum	Thermo Fisher Scientific, New Zealand	
Prolong Anti-Fade Gold Mounting Reagent	Invitrogen™, USA	

2.0.3 Reagent preparation

2.0.3.1 Preparation of KA

Serial dilution was performed to obtain 0.5 μM of KA. 10 mL of UltraPure™ DNase/RNase-Free Distilled Water was added into the KA monohydrate (10 mg) contained vial. The vial was shaken vigorously and vortexed to ensure KA monohydrate dissolved thoroughly, concentration of KA solution is 0.04 mM. Then, 12.5 mL of 0.04 mM of KA was diluted to 10 μM of KA in 50 mL UltraPure™ DNase/RNase-Free Distilled Water (refer to Appendix 1 for the calculation). 10 μM of KA was stored at -20 ℃ for subsequent use. On the day of KA treatment, 0.5 μM of KA was freshly prepared by diluting 2.5 mL of 10 μM KA solution to 47.5 mL of Neurobasal complete media (Bevinahal *et al.*, 2014).

2.0.3.2 Phosphate buffer (PB)

Phosphate buffer is prepared according to the proportion of components shown in Table 2.3. 800 mL of distilled water was heated to 60 ℃ then 2.76 g Sodium Phosphate Monobasic (NaH_2PO_4) and 2.845 g Sodium Phosphate Dibasic (Na_2HPO_4) was added in. The solution is remained heated and stirred until the solution turned clear. The solution was then topped up to 1000 mL using deionized distilled water then autoclaved and store at 4 ℃ for subsequent use.

Table 2.3 The composition of PBS.

Component	Gram/Litre
NaH_2PO_4	2.76
Na_2HPO_4	2.845

2.0.3.3 Preparation of 4 % Paraformaldehyde (PFA)

40 g of PFA added into the beaker contained with 400 mL of distilled water at 60 °C. Next, a pellet of anhydrous NaOH pellet was added to the solution and stirred until it turned colourless. Then, the solution is transferred and filtered into the 1000 mL flat-bottomed flask using filter funnel. 400 mL of PB is then added into the flat-bottomed flask and tested the pH value using pH paper. NaCl and HCl solution were used to neutralise the final pH value to pH 7. The solution was topped with deionised distilled water to the 1000 mL. The PFA is autoclaved at 121 ºC and then stored at 4 °C for subsequent use.

2.0.3.4 Preparation of 0.3 % Triton X solution

150 µL of Triton X was added to 49.85 mL of D-PBS filled stock bottle and stored at 4 °C for subsequent use.

2.0.3.5 Preparation of 5 % goat serum

5 mL of goat serum was added into 95 mL of D-PBS filled stock bottle and stored at 4 °C for subsequent use.

2.0.4 Antibodies

Primary antibodies and secondary antibodies were used in the experiment. The primary antibodies were purchased from Abcam, UK whereas the secondary antibodies were purchased from Invitrogen™, USA. The antibodies used are listed as showed in the Table 2.4.

Table 2.4 List of antibodies

Material	**Manufacturer**	**Application**
MAP2 Monoclonal Antibody (M13)	Invitrogen™, USA	Immunostaining
Anti-GABA A Receptor alpha 1 antibody (ab94585)	Abcam, UK	
Anti-BDNF antibody (ab205067)	Abcam, UK	
Anti-TrkB antibody (ab18987)	Abcam, UK	
Anti-IP3 receptor antibody (ab5804)	Abcam, UK	
DAPI Nucleic Acid Stain	Invitrogen™, USA	
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen™, USA	
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen™, USA	
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Invitrogen™, USA	

2.0.5 Buffer solution

Several buffer solutions were used for different applications. Hank's Balanced Salt Solution was used to rinse the culture plates during PLL coating process whereas Dulbecco's Phosphate Buffered Saline (D-PBS) was used to rinse the cell culture plate seeded with neuron during immunostaining processes. The HBSS buffer solution provide a favourable condition with relatively higher glucose level compared to D-PBS. This reduced the stresses in the neuron and increase the chances of producing a healthier neuron culture. Ultrapure™ DNase/RNase-Free Distilled Water was used in PLL cell coating and used to fill in water reservoir pan in CO_2 incubator. The buffer solutions used are listed as below (Table 2.5).

Table 2.5 List of buffer and solution

Materials	**Manufacturer**	**Application**
Hank's Balanced Salt Solution, HBSS	Gibco™, USA	Cellular work
Dulbecco's Phosphate-Buffered Saline, D-PBS)	Gibco™, USA	Cellular work, Immunostaining
UltraPure™ DNase/RNase-Free Distilled Water	Gibco™, USA	Cellular work

2.0.6 Equipment and consumables

List of equipment and consumables used in the experiments were listed in the Table 2.6 as shown below.

Table 2.6 List of equipment and consumables

Equipment	**Manufacturer**	**Application**
Countess® Automated Cell Counter	Invitrogen™, USA	
CO_2 incubator	NuAire Inc, USA	
Inverted microscope	Carl Zeiss, Germany	Cellular work
VariosKan 2000	Thermo Fisher Scientific, USA	
Confocal Microscope	Carl Zeiss, Germany	
Evos FL Auto 2 Cell Imaging System	Thermos Fisher Scientific, USA	Immunostaining
Microcentrifuge		
Sorvall Legend Microcentrifuge	Thermo Scientific, Germany	
Centrifuge 5424	Eppendorf, Germany	
Microspin	Eppendorf, Germany	Cellular work
Cultureware	Greiner Bio-One, Austria	
6-well plate		
96-well plate		
Glass coverslip, 25mm^2 (round)		
Filter paper	Sigmal-Aldrich®, USA	
Countess® Cell Counting Chamber Slides	Invitrogen™, USA	
Centrifuge tube		
50mL centrifuge tube	Greiner Bio-One, Austria	
15mL centrifuge tube		
1.5mL microcentrifuge tube		