Epigenetic Involvement of GluR2 Regulation in Epileptogenesis

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Abstract

Epilepsy is one of the most common neurological disorders characterized by recurrent seizures. Currently, the underlying mechanisms are not well understood and therapies only serve to relieve the symptoms. A single episode of seizure can trigger epileptogenesis, a process in which the brain undergoes network reorganization including neurodegeneration and sprouting of axons. The mechanisms linking the first seizure to development of epilepsy are currently unknown. Interestingly, changes in neuronal circuitry in epilepsy are accompanied by chronic alterations in the normal brain gene expression profile. Epigenetic mechanisms, including DNA methylation and covalent histone modifications stably program the genome during gestation. However, recent studies suggest also that epigenetic mechanisms might be involved in modifying genome function in response to environmental stimuli. We therefore hypothesize that a single seizure can disrupt normal epigenetic programming in the brain, which results in altered gene expression profiles that drive the network reorganization events.

In this study, we used *in vitro* and *in vivo* models of temporal lobe epilepsy (TLE) by kainic acid treatment to test whether DNA methylation changes are associated with epileptogenesis. DNA methylation is a covalent modification of DNA by adding a methyl group on the 5' position of cytosine by DNA methyltransferases. We focused our analysis on DNA methylation because of its importance role in gene regulation.

Indeed there is an overall inverse correlation between DNA methylation of regulatory regions of genes and gene expression. We closely examined the DNA methylation changes associated with the promoters of the *GRIA2* gene (codes for glutamate receptor ionotropic AMPA 2 subunit), which has been demonstrated to be down-regulated in epilepsy and to be highly implicated in hyper-excitable neuronal circuitries. We detected rapid hypermethylation in *GRIA2* after a 2-hour period of epileptiform activity in the *in vitro* model. Similar changes in *GRIA2* DNA methylation were also observed in our *in vivo* model 10 weeks post-kainic acid injection. We also observed a significant positive correlation between the number of seizures recorded by video-EEG and severity assessed by Racine scale and the average *GRIA2* DNA methylation.

Epileptogenic insults induced by kainic acid treatment led to rapid DNA methylation changes in *GRIA2* gene. This result suggests that alterations in DNA methylation may serve as a molecular memory of the insult, which can lead to the progressive changes in gene expressions, thus contributing to the development of epilepsy as well as the maintenance of an epileptic neuronal circuitry.

Résumé Français

L'épilepsie est l'une des maladies neurologiques les plus fréquentes, caractérisée par des crises épileptiques répétées et chroniques. Les mécanismes sous-tendant les troubles neurologiques associés à la maladie sont encore mal compris et seuls des traitements symptomatiques sont actuellement disponibles. Une seule crise épileptique peut induire un processus d'épileptogenèse durant lequel une réorganisation des circuits neuronaux s'effectue, incluant une neurodégénérescence et un bourgeonnement anormal des axones. Les mécanismes conduisant au développement de la maladie épileptique en tant que telle à partir d'un premier épisode épileptique sont encore inconnus. De façon intéressante, les réarrangements des circuits neuronaux observés dans l'épilepsie sont accompagnés de changements stables de schémas d'expression de gènes. Les mécanismes épigénétiques, incluant la méthylation de l'ADN ou les modifications covalentes des histones, permettent une régulation stable des schémas d'expression des gènes se mettant en place durant la gestation. Cependant, de récentes études suggèrent que ces mécanismes épigénétiques permettent également une réorganisation des schémas d'expression de gènes en réponse à des stimuli environnementaux. Nous avons alors émis l'hypothèse qu'un seul épisode épileptique peut perturber les profils épigénétiques cérébraux normaux, aboutissant à des schémas d'expression de gènes altérés et aux réorganisations cérébrales caractéristiques de l'épilepsie.

Lors de cette étude, nous avons utilisés des modèles *in vitro* et *in vivo* de l'épilepsie du lobe temporal (TLE), par traitements au kaïnate, afin de tester si des changements de méthylation de l'ADN sont associés au processus d'épileptogenèse. La méthylation de l'ADN est un processus épigénétique dans lequel les bases cytosines peuvent être modifiées par l'addition d'un groupement méthyle lors d'une réaction catalysée par des ADN méthyltransférases. Nous avons focalisé notre étude sur l'étude des changements de méthylation de l'ADN en raison de son rôle important dans la régulation de l'expression des gènes. En effet, le niveau de méthylation de régions régulatrices de l'ADN telles que les promoteurs est corrélé négativement au niveau d'expression génique. Nous avons en particulier mesuré les modifications des niveaux de méthylation des promoteurs du gène *GRIA2* (codant pour la sous-unité 2 du récepteur gutamatergique ionotropique AMPA), qui est sous-exprimé dans l'épilepsie et dont la protéine est fortement impliquée dans l'hyperexcitabilité neuronale observée dans les crises épileptiques. Nous avons mesuré une hyperméthylation du gène *GRIA2* à la suite d'une période de 2 heures d'activité épileptiforme dans le modèle *in vitro*. Des modifications similaires ont également été observées dans le modèle in vivo, 10 semaines après une injection intracérébrale de kaïnate. Nous avons également observé une corrélation positive significative entre le nombre de crises épileptiques, détectées par Electro-Encéphalogramme Vidéo, la

sévérité des crises, évaluée grâce à l'échelle Racine, et le niveau moyen de méthylation du gène *GRIA2*.

Les crises épileptiques, induites par un traitement au kaïnate, conduisent à des changements rapides des niveaux de méthylation du gène *GRIA2*. Ce résultat suggère que des modifications des schémas de méthylation de l'ADN pourraient être un mécanisme moléculaire de mémorisation des crises épileptiques, conduisant à des changements progressifs d'expression de gènes et contribuant au développement de l'épilepsie et au maintien de circuits neuronaux anormaux.

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List of Abbreviations

AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
BrdU	5-bromo-2'-deoxyuridine
CA	Cornu Ammonis
CBP	CREB binding protein
CNS	Central nervous system
DG	Dentate gyrus
DNMT	DNA methyltransferase
EGF	Epithelial growth factor
GABA	γ-Aminobutyric acid
GluR2	Glutamate Receptor, ionotropic AMPA 2 (protein)
GR	Glucocorticoid Receptor
GRIA2	Glutamate Receptor, ionotropic AMPA 2 (gene)
HDAC	Histone deacetylase
HKA	High convulsive, kainic acid-treated
KA	Kainic acid
LKA	Low convulsive, kainic acid-treated
lncRNA	Long non-coding RNA
LTD	Long-term depression
LTP	Long-term potentiation
MBD	Methyl-binding domain
MeCP2	Methyl CpG-binding protein 2
miRNA	Micro RNA
NMDA	N-Methyl-D-aspartic acid
RNAi	RNA interference
SAH	S-adenosyl-L-homocysteine
SAHA	Suberoyl anilide hydroxamic acid
SAM	S-adenosyl methionine
SE	Status epilepticus
TLE	Temporal lobe epilepsy
TSA	Trichostatin A
VPA	Valproic acid

Introduction

Epilepsy is the second most common neurological disorder affecting approximately 0.8% of the world's population [1]. Despite the prevalence of epilepsy, however, the underlying mechanisms of the disorder are not fully understood. About 30% of people diagnosed with epilepsy do not respond to any of the existing treatments. Currently, there is neither a cure nor preventive diagnostics for epilepsy. The drugs that are available only serve to relieve symptoms and drug resistance often develops. Therefore, there is an urgent need for new therapeutics with novel mechanisms of action.

Epilepsy is a very complex disorder with many possible triggers such as genetic mutation, traumatic brain injury and fever [1]. Epileptogenesis is the process in which normal brain tissues undergo a series of neuronal reorganizations, thus becoming capable of generating spontaneous neuronal activities that result in seizures. This process is known as the latent period, in which a series of cellular and molecular changes happen such as neurogenesis, neurodegeneration, aberrant axonal sprouting, changes in receptor composition and gliosis which ultimately lead to the reorganization of neuronal circuits in the brain [1-3]. Recently, increasing evidence suggests the involvement of epigenetics in epilepsy, as epigenetic processes are responsible for both short- and long-term gene expression programming. Epigenetics plays a vital role in the normal development and functioning of the CNS by regulating the distinct gene expression programs [4].

The hippocampus is of great interest in epilepsy studies as it has been shown to be important in the generation of seizures; it is often the seizure foci [5, 6]. In addition, animal studies have

shown to easily induce seizures in this structure with low-electrical stimulation [7].

Epigenetic aberrations could potentially result in reprogramming of critical gene circuitries which would in turn alter normal brain physiology. Epilepsy is characterized by significant changes in gene expression [1, 8] that are probably behind the neuronal network reorganizations that permanently alter brain physiology such as mossy fiber sprouting and neurogenesis. For instance, some of the most widely studied gene expression changes are those in the neurotransmitter receptor families like GluR2 (Glutamate Receptor, ionotropic AMPA 2) [1, 9]. Chromatin modifying drugs were shown to reverse induction of genes during experimental *status epilepticus* (SE) in animal models implicating epigenetic mechanisms in these models [10, 11]. Epigenetic changes have also been observed in other neurological disorders that involve an increased risk of spontaneous seizures such as Rett Syndrome [12]. Therefore, these previous findings point to the possibility that epigenetic reprogramming might play a casual role in the molecular pathology of epileptogenesis. However, previous studies only examined histone modifications and therefore the state of DNA methylation has remained unexplored.

GRIA2 gene was targeted for this study for several reasons. First, a previous study had shown that a seizure could alter histone acetylation at this gene [13]. Second, GluR2 (the protein coded by the gene *GRIA2*) down-regulation has been well documented in several epilepsy studies (examples seen in [14], [15]). Lastly, down-regulation of GluR2 could result in increasing populations of Ca^{2+}-permeable AMPA receptors that contribute to maintenance of hyper

excitability and pathophysiology.

Literature Review

Epilepsy and the Hippocampus

Epilepsy is a group of complex and chronic neurological disorders characterized by recurrent and unprovoked seizures. There are many types of epilepsy that are classified according to the affected region and extent of its spread, as well as the clinical symptoms. A large proportion of patients suffering from epilepsy have seizures generated in mesial temporal lobe [16], which includes the hippocampus and the adjacent perirhinal, entorhinal and parahippocampal cortices.

The hippocampus has been of great interest to researchers in epilepsy studies as it is often the epileptic focus; electrographic abnormalities within this structure have often been detected by intracerebral recordings [5]. Moreover, the surgical removal of this structure in patients often leads to reduced or abolished seizures [6]. The hippocampus is positioned in the medial temporal lobe in the brain. It contains two histologically distinct regions: Ammon's horn or Cornu ammonis (CA) and the dentate gyrus. The layer of pyramidal cells is mainly made up of excitatory glutamatergic pyramidal cells. This region is further classified into two main subfields by their morphology: CA3 and CA1. Compared to CA1 pyramidal cells, those from CA3 are larger in size and they all have different inputs and outputs. CA3 cells are innervated by mossy fibers originated from the dentate gyrus, entorhinal cortex II whereas CA1 cells receive inputs from entorihinal cortex III and CA3 cells.

The dentate gyrus (DG) consists of three layers: the first two, the molecular layer (mainly occupied by dendrites originating from other DG layers or other regions) and the principal cell layer (a dense layer of granule cells), together they form a structure called fascia dentate that engulfs the

third layer, the polymorphic cell layer. The primary cell type in the DG is granule cell. Granule cells send axons that innervate other areas of hippocampal fields like CA3 [17]. There are variations in the neurons found in the hippocampus that have different properties due to their morphological characteristics, connectivity, and age [18]. Throughout life, the hippocampal neuronal network continuously undergoes re-organization via rearrangements of dendrites and synapses, as well as neurogenesis. It is thought that neurogenesis in the hippocampus, which only occurs in the DG is important for the modulation and fine-tuning of the existing neuronal circuitries [19]. Newly formed neurons in the adult brain are different from the existing granule cells; they have different electrophysical properties from the existing mature neurons. For example, GABAergic transmission exerts an excitatory effect in newly formed neurons, as opposed to the inhibitory effect in mature neurons. In addition, T-type calcium channels are induced which enhances the initiation of fast action potentials. These properties of newborn neurons increase the excitability which results in a lowered threshold of LTP (long-term potentiation, a form of long-lasting augmentation in synaptic signaling between neurons that improves synaptic strength and enhanced synaptic plasticity [20-23]).

During epileptogenesis, researchers have observed a series of changes in fundamental neuronal properties at the cellular level such as receptor composition, increased excitatory but decreased inhibitory transduction and re-enforced connections between neurons. Immediately after the seizure, there is release of neurotransmitters, especially glutamate. This is followed by the

activation of ion channels, which result in an influx of calcium causing a series of downstream effects. An example is post-translational modifications of downstream effectors that lead to activations of the immediate early genes (a group of transcription factors), which further induce their target genes to modulate both short and long-term neuronal modifications. At the same time, neuronal injuries also occur and can lead to cell death due to excitotoxicity.

Following the acute responses, there are chronic remodeling events that eventually lead to the onset of recurrent seizures: excitatory kainite receptor-mediated mossy fiber sprouting that innervate new targets, neurogenesis, angiogenesis and reactive gliosis [24]. Ultimately, the brain becomes predisposed to synchronous neuronal activities, which lead to epileptic attacks.

The Epigenome

Components of the epigenome

The genome contains critical programs required for development and growth, and the epigenome is responsible for controlling and executing these sophisticated regulatory programs. The epigenome consists of the chromatin and its covalent modifications on histones, as well as DNA methylation on the 5' position of the cytosine ring. In addition, researchers have recently discovered a new class of epigenetic regulations by non-coding RNAs such as miRNA (micro RNAs) and lncRNA (long non-coding RNA). miRNAs are thought to play roles in neural differentiation and plasticity [25], whereas lncRNAs have implications in the regulation of chromatin remodeling and post-transcriptional RNA processing [26]. Through the combinations of various epigenetic mechanisms, the epigenome imposes long-term programming to regulate every aspect of an

organism, such as development and aging, homeostasis and transgenerational inheritance. Cell specific epigenetic states are established during development which allows for the emergence of several cell type specific gene expression profiles.

The epigenome also serves as the cell's "molecular memory" by transferring information from one generation of cells to the next, maintaining the cell's properties as well as those of the tissue. The established epigenetic marks were previously believed to be static after differentiation is completed and to be copied faithfully during cell division. However, recent studies have proven that the epigenome is highly dynamic. A portion of the established marks are dynamic and flexible throughout life, in response to environmental signals such as food, chemicals, maternal care, stress, and social behaviors [27-29]. The epigenome serves as an interface between the static genome in the nucleus and cytoplasmic signaling cascades, thus allowing responses to changing environments by delineating new patterns of gene transcription.

Furthermore, epigenetic aberrations can have similar effects as genetic polymorphisms, resulting in variations in gene function [30]. The epigenetic mechanisms regulate gene expression through the control of the chromatin structure, thus determining the openness of genome targets to transcriptional machineries and co-activators. Genes that are inaccessible are silenced and those that are accessible are transcribed.

The histone and chromatin structure

The most fundamental unit of chromatin is a nucleosome. It is comprised of DNA wrapped

around an octamer histone core composed of histone proteins (two of each H2A, H2B, H3, and H4). In addition to packaging DNA in the nucleus through supercoiling by histones, a nucleosome confers local and global chromatin architecture via histone tail modifications and higher-order chromatin remodeling. Histone modifications are catalyzed by enzymes that covalently modify the N-terminal domains via methylation, acetylation, phosphorylation, ubiquitylation, biotinylation, DP-ribosylation and SUMOylation. By changing DNA-histone electrostatic interactions, histone modifications have specific functional effects depending on the site modified [31]. Existing histone modifications can affect the occurrence of following modifications either occurring on the same histone or between histones in a nucleosome or even on another nucleosome [31-33]. A well-characterized example of cross-regulation is when epidermal growth factor (EGF) activates the Ras-MAPK pathway. Gcn5 (a histone acetyltransferase) acetylates H3K14 following H3S10 phosphorylation. In addition, phosphorylated H3S10 inhibits H3K9 acetylation. In contrast, when H3K9 is methylated by Suv39hl (a histone methyltransferase), it inhibits H3S10 phosphorylation, as well as G9a (a histone methyltransferase)-mediated methylation on other residues [33, 34].

Different histone modifications or in combination make up the “Histone Code” and together with chromatin-associated proteins like HP1 (heterochromatin protein 1, which aides in gene repression by forming a densely-compacted chromatin structure - heterochromatin), define the transcription profile of a cell [34]. The Histone Code is interpreted via binding of effector proteins triggering downstream functions. Chromatin structure remodeling complex are a series of proteins

that act together to determines the availability of a transcription initiation site by altering the position of nucleosomes around it through an ATP-dependent manner. Chromatin structure remodeling complexes like ATRX in the SWI/SNF family of proteins function not only in transcriptional regulation, but also in heterochromatin formation, DNA repair, and chromatin segregation [35]. However, the focus of the project is not on histone modification and chromatin remodeling, and therefore they will not be further discussed.

DNA methylation

DNA methylation has not been explored to the same extent as histone modifications have been in the areas of neurological disorders, particularly in epilepsy. DNA methylation is an essential epigenetic machinery in vertebrates and plants, mainly occurring at cytosines that are part of the CG di-nucleotide sequence. It is mediated by DNA methyltransferases (DNMTs) that transfer a methyl group from methyl-donor S-adenosylmethionine (SAM) to the 5’ position of a cytosine ring. It is a repressive mark where the actively transcribed region of the chromatin is associated with hypomethylated DNA, whereas inactive chromatin is associated with hypermethylated DNA. DNA methylation is important in X-chromosome inactivation and genomic imprinting during organism development, and it maintains satellite repeat sequences and silences endogenous retrovirus.

Out of all the DNMTs that have been discovered, there are three that are most functionally important: DNMT1, 3a and 3b. Knockout mice of any of the three DNMTs are not viable [28]. DNMT1 is classified as a maintenance methyltransferase, as its preference for hemi-methylated

DNA allows the maintenance of methylation patterns during DNA replication in mitosis. DNMT3a and 3b are classified as d*e novo* methyltransferases; they have equal preference for methylated and unmethylated DNA and they are able to methylate new sites. However, new evidence has shown that these rules do not always apply. Studies utilizing DNMT1, 3a or 3b knockout cell lines showed that DNMT1 alone cannot maintain methylation levels at certain repeat regions. Likewise, without the presence of DNMT1, DNMT3a and 3b are not able to effectively *de novo* methylate DNA [36]. Abnormal *de novo* DNA methylation can lead to hypermethylation of tumor suppressor promoters such as those involved in cell cycle regulation (p16, Rb) and DNA damage repair (BRCA1, a E3 ubiquitin-protein ligase and MGMT, 6-O-methylguanine-DNA methyltransferase), which can lead to cancer progression [37].

DNA methylation participates in the repression of gene transcription in two major ways: first by directly interfering with transcription factor binding to its recognition element in the promoter region of a gene [38]. Secondly through the recruitment of methylated DNA-binding (MBD) proteins such as MeCP2 (methyl CpG binding protein 2). MeCP2 acts to recruit repressor complexes such as Sin3A (a transcription regulator and histone deacetylase) and other histone deacetylases [39], which compact the chromatin and leads to transcriptional silencing. Other MBDs such as MBD1 and MBD2 act through similar mechanism and repress transcription. MBD3 indirectly represses gene expression through its association with repressive chromatin remodeling complexes such as NuRD (a transcriptional repressor complex that has not only ATP-dependent chromatin remodeling

activity, but also histone deacetylase activity), which binds to methylated DNA via MBD2. Some experimental evidence suggests that there is replication-independent DNA demethylation followed by TSA (trichostatin A, a histone deacetylase inhibitor) treatment which increases histone acetylation [40, 41]. MBD2 has been proposed to have DNA demethylase activity [42].

Epigenetic Regulations in the Brain

Involvement in Cognition and Behavior

Epigenetic processes play critical roles in synaptic plasticity, which is required for learning and memory. Synaptic plasticity describes the ability of neurons to strengthen or weaken their synapses with other neurons following activation [43]. During formation of long-term memory, accurate regulation of gene expression at both transcriptional and translational levels is required, thus affecting the number of receptors located in the synapse and as well as the amount of neurotransmitters released [44-47].

Studies using the marine mollusk *Aplysia* have provided insights into the mechanisms of synaptic plasticity associated with memory acquisition. Upon the induction of synaptic sensitization (long-term facilitation, a form of synaptic plasticity), CREB1-mediated up-regulation of the C/EBP gene was observed. The study reported increased CBP (CREB-binding protein, which is also a histone acetyltransferase) binding to the C/EBP gene promoter, which led to H3K14 and H3K8 acetylation in the region. In contrast, by inducing a form of synaptic desensitization (long-term depression), C/EBP down-regulation mediated partly via HDAC5 (histone deacetylases 5) recruitment was observed [48].

Similar phenomena are seen in mammals; mice with defective CBP exhibited reduced level of acetylation at H2B as well as transcription-dependent defects in the late phase of LTP induction. However, there were no abnormalities seen in the early transcription-independent phase of LTP and no alteration in basal synaptic transmission [10]. These observations explain the impaired long-term memory but unaffected short-term (transcription-independent) memory observed in these mice when challenged with fear conditioning and object recognition tasks [10, 49, 50]. Moreover, through administration of SAHA (suberoyl anilide hydroxamic acid, a histone deacetylases inhibitor), which compensates for CBP's acetyltransferase activity, deficits in long-term memory were improved and late phase LTP was enhanced [10]. Also, another study has shown that the administration of trichostatin A or sodium butyrate (HDAC inhibitors) to wild type mice enhanced the induction of hippocampal LTP and spatial memory and this was associated with increases of H3 and H4 acetylation [51, 52], for instance at CREB-mediated genes like Nr4a1 and Nr4a2 (can either homo or heterodimerize to activate downstream targets. They are involved in memory formation and consolidation) [52]. These data in combination suggest that the impairment in synaptic plasticity in CBP deficient mice was mainly caused by the loss of epigenetic activity of CBP [10, 49, 50, 52].

DNA methylation is also an important contributor to brain plasticity. First, studies have shown that the depolarization of neurons triggers the up-regulation of BDNF and demethylation at its promoter regions [53, 54]. When inhibitors of DNMTs (5-azaC and zebularine) were applied to mice

hippocampal slices, the induction of LTP was blocked. [55, 56]. Furthermore, MBD1 knockout mice exhibit deficits in spatial learning and reduced LTP induction in the dentate gyrus, where MBD1 is normally expressed at high levels [55]. Consistent with the previous results, mice with conditional knockouts of DNMT1 and 3a in excitatory neurons of the forebrain exhibit loss of LTP and enhanced LTD induction. The double knockout mice in DNMT1 and DNMT3A have deficits in learning and memory when tested in the Morris water maze[36].

A more direct link to cognition was demonstrated by examining epigenetic modifications of genes, namely PP1 (phosphatase 1, a suppressor of hippocampal LTP and memory formation [57, 58]) and reelin (a member of extracellular matrix proteins, which is important for neuronal migration during development and in synaptic plasticity [58]). During the contextual fear conditioning test, PP1 was rapidly methylated and transcriptional silencing was observed. In contrast, reelin was rapidly demethylated and transcriptionally activated [58]. This demonstrates that DNA methylation is involved in cognition. In addition, DNMTs were up-regulated after fear conditioning the animals. By treating the animals with 5-azaC (an inhibitor of DNMTs), PP1 hypermethylation was reversed and transcription level was restored, which resulted in a blockade of memory formation in the animals when tested [58]. This evidence shows that synaptic plasticity, which is important for learning and memory requires the dynamic modulations of specific epigenetic mechanisms in fully developed post-mitotic neurons.

The dynamic epigenome and influence on brain development

Epigenetic regulation has considerable impact throughout brain development [59]. Perhaps the most stunning example of such regulation is how early life experience can influence and shape each individual's behavioral outcomes through epigenetic programming. In rodents, early postnatal day experience is associated with alterations in various receptor pathways. Experience such as maternal care by means of licking and grooming and arched-back nursing influence the offspring's HPA activity in response to stress. Offspring reared by high maternal care dams have higher expression of glucocorticoid receptor (GR) and reduced stress responsiveness in the hippocampus compared to those reared by dams that provided low maternal care [27, 60]. An analysis of the GR promoter found that increased methylation at the promoter of GR 1-7 exon is responsible for a higher level of GR, and that this epigenetic profile persists into adulthood [27]. In addition, low maternal care is associated with lower H3K9 acetylation at GR 1-7 and reduced transcription factor NGFI-A (nerve growth factor-inducible protein A). However, the infusion of HDAC inhibitor TSA abolished the effect of maternal care on HPA stress response, GR expression, DNA methylation, histone acetylation, and NGFI-A binding [27, 28].

Similar effects are seen in humans; early life adversity such as childhood abuse and neglect has a tremendous impact on elevating activity in HPA axis in response to stress, thus increasing the risk of suicide. Adverse childhood experience has an impact on the expression of GR via increased DNA methylation at its promoter, thereby impairing the feedback inhibition of high-circulating glucocorticoid in response to stress [28]. Studies examining various neurotransmitter systems in

rodents provided further insights into the mechanisms underlying how social experience influence brain development and as a consequence alter behavior; alterations in the activities and properties of neural pathways such as GABAergic, glutamatergic and dopaminergic pathways contribute to the shaping of neurobiology and ultimately emotionality, cognition and behavior [61-68]. These modulations in response to the social environment are thought to be regulated by epigenetic mechanisms [59].

Neurological Disorders and Cognitive Dysfunction

Defects in epigenetic regulation can lead to CNS disorders and often, cognitive impairment. Specific epigenetic regulations are important for certain developmental processes in the CNS. Interference with these processes results in abnormal phenotypes such as mental retardation and intellectual disability. Pathologic disruption of proper epigenetic regulation could be caused by genetic mutations of epigenetic regulators (in Rett syndrome, Rubinstein-Taybi syndrome, Alzheimer's disease, Huntington's disease). Alternatively epigenetic aberration could directly cause disease (Fragile X syndrome, schizophrenia, Angelman syndrome) [69-73]. Interestingly, many of these disorders are accompanied by epilepsy [74-76].

Evidence such as that outlined above suggests the involvement of epigenetic mechanisms in the mediation of the pathophysiology of epilepsy. First, inhibiting DNMTs in hippocampal neurons led to reduction in neuronal excitability and activity [77]. In addition, in both human and experimental models of temporal lobe epilepsy, epigenetic modifying drugs were demonstrated to

alter expressions of many genes that are directly linked to the onset - and also progression - of the disorder [78]. For example, the levels of reelin are decreased in specimens of human temporal lobe epilepsy (TLE) and are linked to granule cell dispersion, which is a common characteristic of the dentate gyrus in TLE [79]. An examination of the reelin promoter revealed that the methylation levels were higher in specimens of TLE than control and the increased methylation had significant correlation to the occurrence of granule cell dispersion[78].

Changes in histone acetylation of BDNF and GluR2 were observed in patients and as well as experimental models of epilepsy [11, 80, 81]. It was thought that the effect was caused by neuronal restrictive silencer factor (NRSF, binds and silences neuronal genes) mediated regulation, as these genes contain NRSF binding site and it is rapidly induced in the hippocampus following a seizure [81]. In support of this finding, NRSF was found to be associated itself with some epigenetic-modifying enzymes such as KDM5c (histone demethylase) and certain HDACs [81, 82]. Furthermore, treatment with HDAC inhibitors such as valporic acid (VPA) or TSA was able to restore the expression of these genes [81]. Interestingly, NRSF is involved in the regulation of many genes that are implicated in epileptogenesis. These include growth factors, neurotransmitter receptors, ion channels and gap junctions [82]. Moreover, HDAC inhibitor treatments abolished the seizure-induced neurogenesis after kainic acid-induced seizures, but failed to prevent seizure-induced neurodegeneration [81]. On the other hand, miRNAs are also thought to contribute to the progression of the disorder; a study found that after 24 hours of kainic acid-induced seizure,

certain miRNAs had differential changes in expression, namely up-regulations of miRNAs 10 and 13, and down-regulations of 18 and 21. The differential changes in miRNA levels correlated with their corresponding target mRNA levels. Many of the target genes are implicated in pathways like cell morphology, cell death and organismal development [82]. This evidence shows that there are aberrant epigenetic regulations in the onset and progression of epilepsy, but that the precise mechanisms need to be more carefully investigated.

Glutamatergic Transmission and Roles of GluR2 in Epilepsy

Glutamatergic transmission is not only the major excitatory synaptic transmission in CNS, it is also involved in neuronal development [83] and the maintenance of several forms of synaptic plasticity, including LTP and LTD (long-term depression, a weakening in the transmission efficacy of synapses) [84]. There are two groups of glutamate receptors classified by their structures. Metabotropic glutamate receptors are G-protein coupled and are further divided into three groups. Group I receptors are typically expressed postsynaptically and they activate phospholipase C, which produces secondary messengers diacylglycerol and inositol triphosphate. Group I receptors can also increase excitatory transmission by activating other cation channels. Group II and III receptors are typically found in presynaptic terminals and are inhibitory auto-receptors. They can be activated when the levels of glutamate rise during a repetitive stimulation of synapses. Receptors in Groups II and III negatively regulate adenylyl cyclase, which leads to reduced cAMP.

On the other hand, ionotropic glutamate receptors are ligand-gated cation channels and they

contribute to excitatory action by increasing Na^+, K^+ and sometimes Ca^{2+} conductance, depending on the receptor composition. Ionotropic glutamate receptors are also subdivided into three subgroups depending on their activation in response to the binding of selective agonists: NMDA (N-Methyl-D-aspartic acid), kainic acid and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid). All the receptors are composed of four subunits. NMDA receptors are composed of NR1, which has various splice variants and a selection of NR2A, B, C or D subunits that determine the electrophysiological properties of the formed receptor, such as the duration of channel opening. A very distinct feature of NMDA receptors is that they have a voltage-sensitive Mg^{2+} block. The block is only removed during a partial depolarization of the membrane potential. Kainate receptors are highly expressed in certain structures, such as the hippocampus, cerebellum and spinal cord. They are composed of GluR5 to GluR7 subunits as well as KA1 and KA2. AMPA receptors are heterotetramers composed of GluR1 to GluR4. AMPA receptors are present in all neurons. The permeability of the Ca^{2+}receptor is determined by the GluR2 subunit. GluR2 mRNA undergoes RNA editing, where a glutamine (Q) at the M2 domain, which forms the channel with other subunits, is replaced with an arginine (R) residue. By changing to the positively-charged arginine, the passage of Ca^{2+} ions is limited [85]. Thus, the presence of a GluR2 subunit in the receptor regulates its Ca^{2+} permeability [86]. There is also alternative exonal splicing of the AMPA receptor subunits that contributes to the receptor's wide range of functions. The flip/flop sequence is an example of interchangeable exons. The flip/flop splicing affects the rate

desensitization, re-sensitization, and channel opening of the incorporated receptor [87, 88]. It has been demonstrated in animal models as well as in epilepsy patients that the ratio of the flip/flop expression pattern of AMPA receptor subunits was increased in the hippocampus [89-91]. This in turn enhances the response to glutamate, allowing the synapses to operate at a higher efficiency, which increases the occurrence of paroxysmal discharge observed during epileptiform activities [91]. Excessive levels of intracellular Ca^{2+} can cause excitotoxicity to neurons and result in cell death [85, 92]. This can be caused by increased expression of Ca^{2+} permeable AMPA receptors, due to GluR2 subunit down-regulation [85, 93]. In agreement with this hypothesis, as mentioned earlier, the expression of the GluR2 subunit is low in the developing brain, which contributes to an increased susceptibility to seizures [94]. Also, in animal models, GluR2 was shown to be down-regulated prior to neuronal cell death [14, 15]. Furthermore, transgenic mice harboring a non-functional GluR2 subunit through the knockout of the GluR2 mRNA editing process displayed a significantly increased AMPA receptor-mediated calcium current in the hippocampal neurons, which corresponds to enhanced seizure susceptibility in these mice [95]. Moreover, *in vivo* knockdown of GluR2 in mice demonstrated its age-specific effects on seizure control, hippocampal neuron survival and synaptic plasticity [96]. Collectively, this experimental evidence implicates GluR2 in epileptogenesis. Besides GluR2, other members of glutamatergic system also have roles in seizure generation as shown by studies utilizing receptor agonists and antagonists. Ionotropic receptor antagonists (more specifically, NMDA and AMPA) like D-CPPene, MK-801 and GYKJI-52466

cause seizure control in animal models [97]. Metabotropic glutamate receptors are also potential therapeutic targets for anticonvulsants; antagonists of Group I receptors have an anticonvulsant effect, whereas agonists of Group II, III receptors have mixed pro-convulsant and anticonvulsant effects [97].

Project Rationale

Recent evidence has pointed to the possible involvement of aberrant epigenetic regulation in progression of epilepsy. Therefore, we set forth to understand the epigenetic mechanisms that underlie epileptogenesis. In this study, we focused on DNA methylation because it has not been explored in epilepsy. We chose to examine *GRIA2* (Glutamate Receptor, ionotropic AMPA 2) as previous studies have shown that this gene is linked to the maintenance of a hyper-excitable neuronal network and that a seizure could alter the state of histone acetylation of this gene. We hypothesize that a brief seizure can chronically disturb normal DNA methylation programming in the *GRIA2* of the brain which results in changes in gene expression and ultimately contributes to reorganization of neuronal network that is involved in generation of spontaneous activities resulting in seizures.

In order to test our hypothesis, we first used an *in vitro* system of mouse organotypic hippocampal cultures to examine DNA methylation changes in the early stage of epileptogenesis. We treated the cultures with kainic acid under a controlled environment for two hours (before cell death due excitotoxicity could occur) to induce epileptiform activities. Afterwards, *GRIA2* DNA

methylation was assessed with bisulfite pyro sequencing and the transcription level of the gene was checked with qRT-PCR. Hippocampus was chosen as a target tissue because of its importance in seizure generation and has been widely used to study epilepsy. Next, in order to determine whether changes in DNA methylation in *GRIA2,* an *in vivo* model of epilepsy was utilized. Rats were injected with kainic acid to produce epilepsy. Epileptic rats were then monitored for seizure activities and intensities. Rats were then sacrificed for assessment of *GRIA2* DNA methylation at the homologous region to the one examined in mouse. *GRIA2* DNA methylation data and seizure information were paired and analyzed for possible correlation for each individual rat.

Methods and Materials

In vitro mouse organotypic hippocampal slice cultures

Hippocampi from postnatal day 6 BALB/c mice were sliced to 400µm in thickness by McIlwain tissue slicer. Subsequently, slices were immersed in a balanced salt solution to wash away tissue debris and potentially harmful substances such as excitatory amino acids. Each slice was then mounted onto a poly-D-lysine-coated glass cover slide with chicken plasma (Cocalico, New Jersey) and thrombin (Sigma-Aldreich). They were then inserted into a plastic tube containing 500µL media (25% horse serum, 50% Eagle medium, 25% balanced salt solution; pH 7.4). The tubes with slices were maintained in a roller-drum at 36 °C and media was changed weekly. Each organotypic slice was cultured for 3 weeks to allow differentiation and maturation of the neural circuitries. The morphology of the resulting slices was similar to those observed in vivo [98, 99]. During the initial period of cultivation, the slice cultures thinned down to approximately 50 to 100 µm thick allowing higher visualization of neurons and accessibility to individual cells for micromanipulation (Figure 2, shows the morphology of a typical slice culture). There are many advantages of using organotypic hippocampal slice cultures. The structure from the tissue-of-origin is preserved. This is extremely important for our study because epileptiform activity can only occur in a network of neurons. Furthermore, organotypic slice cultures can survive for 4 months in vitro before they start to degenerate, allowing long-term treatment and study. To induced epileptiform activities in the slice cultures, the slices were incubated with kainic acid (6 µM) for two hours before they were assayed for DNA methylation changes.

In vivo rat model

This was done in collaboration with Dr. Nigel Jones from the University of Melbourne, Australia. At 9-10 weeks of age, rats were injected with 5 mg/kg Kainic acid (ip) which induced status epilepticus (SE), a period of sustained seizure activity. Four hours after kainic acid, animals were given an injection of diazepam (5mg/kg ip) to cease their seizures. Control animals received saline (0.9%, 2ml/kg ip) coupled with diazepam. Animals were then left to recover, and over the ensuing weeks, spontaneous recurrent seizures developed in a subgroup of animals, leading to a diagnosis of epilepsy. Kainic acid-induced status epilepticus rat model exhibits very similar properties to clinical human mesial temporal lobe epilepsy [100-102].

At 8 weeks post-SE, all rats were surgically implanted with extradural recording electrodes according to a previously established protocol [103]. Briefly, rats were anaesthetized with isoflurane (5% induction, 2-3% maintenance) and a midline incision was made on the scalp. The connective tissue was removed, and 6 burr holes drilled into the skull. Brass recording electrodes were then gently screwed into the holes, and held in place by dental cement.

At 10 weeks post-SE, all animals underwent 2 weeks of video-EEG recording (Compumedics, Australia) to record the frequency and severity of spontaneous seizures. The severity of seizures was then assessed with Racine scale. At the completion of the 2 weeks recording, animals were rapidly decapitated, and the brains excised and immersed in ice-cold artificial CSF containing (mM): 125 NaCl, 3 KCl, 6 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 10.6 glucose. The hippocampus structure was then microdissected using a dissecting microscope, and the tissue snap-frozen for

DNA extraction and pyrosequencing analysis of DNA methylation of the rat *GRIA2* gene

Bisulfite Pyro Sequencing:

Epitect Bisulfite Kits (Qiagen) were used for bisulfite conversion of DNA as described in the manufacturer's manual. Samples were prepared by performing nested PCR with one of the nested primers carrying a 5' biotin modification. Primers for mouse *GRIA2* gene were: Outside Forward – TGTGGATTGTTTTTGTATTATAGT, Outside Reverse – AAACCCCTTCTCACAAACTTTA; Nested Forward – GGATTGTTTTTGTATTATAGTGTA, Nested Reverse – ACATCCATTCTAAACTACTACC. Primers used for rat *GRIA2* were: Outside Forward – AAAGTAAAAATATTTTTTGAAAGGA, Outside Reverse – ATCTAAAAACCAAATCTACATAACC; Nested Forward – TTTATGATGTAAGTATAATTTTAGGGAAAT, Nested Reverse – TTCAAAAACAATCCACAAACAATAC. All primers (designed against bisulfite-converted DNA) were synthesised by IDT Technologies. PCR conditions consisted of initial denaturation/enzyme activation at 95°C for 3 min, then 40 cycles of 95°C for 30 sec, respective annealing temperature for 30 seconds, 72 °C for 30 seconds, and completed with a final extension step at 72 °C for 4 minutes. Annealing temperatures were: mouse outside PCR 54.8°C; mouse nested PCR 49.6°C; rat outside PCR 50.8°C; rat nested PCR 55.1°C.

Pyro Sequencing was then performed with a PyroMark Q24 machine using the procedure described in the manufacturer's manual. Briefly, nested PCR products were incubated with sepharose beads (GE Healthcare, 17-5113-01) and agitated for 5 minutes. The beads with DNA were then washed in 70% ethanol, and the DNA strands denatured in 0.2M NaOH with each step lasted 5 seconds. They

were then washed again for 10 seconds, and mixed with an annealing solution containing the relevant sequencing primers: Mouse -GTAGATTTGGTTTTTAGATG; Rat region 1 – ATTTTTAATTATAAAAGATGT, Rat region 2 - GTTTTTTTGGGTTATGG. The samples were then processed by the Pyro Sequencer, and the resulting percentage methylation at the targeted CpG sites was calculated with the accompanying software (PyroMark® Q24 Software).

RNA Extraction

Trizol reagent (InVitrogen) was used to extract RNA from mouse organotypic cultures. Four cultures or 100mg of hippocampal tissues were homogenized in 1mL of Trizol by pipetting up and down and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. 0.2 mL of chloroform was then added and mixed for 15 seconds by vortexing then incubated at room temperature for 3 minutes. Afterwards, the mixture centrifuged at 12,000 x g for 15 minutes at 4 °C. Following centrifugation, the upper phase which contains RNA was transferred into a new tube and 0.5 mL of isopropanol was added to precipitate RNA. The resulting mixture was incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for another 10 minutes at 4 °C for precipitation of a RNA pellet. The RNA pellet was washed with 1mL of 75% EtOH by vortexing and centrifuge at 7,500 x g for 5 minutes at 4 °C. The supernatant was then discarded and the RNA pellet was dissolved in 40 µL of RNase-free water.

Real-time Quantitative PCR for Gene Expression Analysis

Extracted RNA was subjected to conversion to cDNA with First Strand cDNA Synthesis Kit for RT-PCR

(Roche). 2µg of RNA was added to a mixture containing 2.0µL of 10x Reaction Buffer (supplied with kit), 4.0 µL of 25mM $MgCl_2$, 2.0 µL of 10mM deoxynucleotide mix, 2 uL of random primers, 1.0 µL of RNase inhibitor and 0.8 µL of AMV reverse transcriptase. The mixture was then mixed by brief vortexing and consolidated by centrifuging. The reaction was incubated in a thermal cycler with the following program: 25°C for 10 minutes, 42°C for 60 minutes and 95°C for 5 minutes and kept at 4°C. After completion of cDNA synthesis, a serial dilution of cDNA at 1:5, 1:10, 1:25, 1:50 and 1:100 was made to generate a standard curve for calculation of mRNA levels in subsequent reactions. For real-time quantitative PCR of gene expression, a Roche LightCycler® 480 Real-Time PCR System was used with LightCycler® 480 DNA SYBR Green I Master for amplifications of target genes. For each reaction, 1µL of cDNA (sample or standard curve sample) was added to a mixture of 10µL SYBR green master, 0.5µL each of forward and reverse primers and 8.0 µL of water. The amplification program was: initial enzyme activation at 95°C for 10 minutes followed by another denaturing step at 95°C for 10 seconds, primer annealing (different for each primer) for 10 seconds, extension at 72°C for 10 seconds for 45 cycles. A triplicate analysis was performed per sample and GAPDH was used as the reference gene. Primer sequences were purchased from IDT Technologies: *GRIA2*: forward –TGGAGTATTCTACATCCTTGTCGG, reverse – GCCCTTGACTTGTAACAGAACTCA; GAPDH: forward: AAATGGTGAAGGTCGGTGTG, reverse: TGAAGGGGTCGTTGATGG. Target gene mRNA levels were analyzed by Roche LightCycler® 480 Software with the use of a standard curve and crossing point of each sample to calculate the relative concentration.

DNA Extraction Procedure

Cultures were incubated either overnight or for 16 hours with 10µL of 20mg/mL Proteinase K (Roche, 03115836001) in 500µL of Lysis Buffer which consisted of 10mM Tris Base (Fisher, BP1521) pH 8.0, 0.4M NaCl (Fisher, SS84-1), 2mM EDTA (Fisher, S312-500), and 1% SDS (Sigma-Aldrich, L3771-100G). Next, add 150uL of saturated NaCl to the mixture and mix by inverting. The mixture was centrifuged at 13,200 rpm for 15 minutes at 4 degrees Celsius and the supernatant was divided into 2 new eppendorf tubes. Next, 150µL of saturated NaCl was added to the mixture and mixed by inverting. The mixture was centrifuged at 13,200 rpm for 15 minutes at 4 °C and the supernatant was equally divided into 2 new eppendorf tubes. Next phenol-chloroform extraction was performed; 330µL of phenol and same amount of chloroform then mixed by inverting. The mixtures were centrifuged at 13,200 rpm for 15 minutes at 4 °C. The aqueous phase, which contained DNA, was transferred into a new eppendorf tube and d 2µL of glycogen (Roche, 10901393001) and 900uL of 95% EtOH were added and incubate at -80 °C for 1 hour. After incubation, the solution was centrifuged at 13,200 rpm at 4 °C for 15 minutes. The supernatant was discarded and DNA pellet was washed with 200µL of 70% EtOH. The contents were mixed by inverting and centrifuged at 13,200rpm for 15 minutes at 4 degrees Celsius. Again the supernatant was discarded afterwards and washed. The DNA pellet was allowed to dry for 10 minutes prior to dissolving in 1x TE buffer (10mM Tris with 1mM EDTA). After dissolving, the DNA solution was treated with 1µL of RNase A (Fermentas, EN0531) and incubated at room temperature for 5 minutes. Then phenol-chloroform extraction procedures was repeated and proceed with EtOH DNA precipitation and DNA wash.

Finally, DNA pellet was dissolved in 1x TE buffer.

BrdU Treatment and immunostaining

(As adapted from Raineteau et al (2004) [104] In brief, organotypic cultures were incubated with BrdU (Sigma-Aldrich, B5002-100MG) in culturing medium for 3 days before fixing with 4% paraformaldehyde overnight (Sigma-Aldrich, 47608-250ML-F). Cultures were then removed from their cover slips. Denaturation of DNA was done with 50% formamide (Sigma-Aldrich, F9037-100ML) at 65°C. Cultures were then washed extensively with phosphate buffer before being permeablized. Anti-BrdU antibody (Fitzgerald, 20-BS17) were applied at 4°C overnight followed by extensive washing. Finally, Alexa 568 conjugated secondary antibodies (Invitrogen, A21099) were applied overnight at 4°C.

Statistical Analysis

Two-way ANOVA was performed to compare the percentage of DNA methylation at each CpG site between treatment groups. For the mRNA expression of GluR2, a Student's t-test compared between treatment groups. For the correlative analysis, the percentages of DNA methylation across the 5 CpG sites at the *GRIA2* gene locus were averaged in each rat to obtain an average percentage of methylation for each animal. The strength of correlation between the number of convulsive seizures experienced and the percentage of DNA methylation was assessed using Pearson's correlation analysis. All analyses were performed using Graphpad Prism software, and statistical significance defined in each case as $p<0.05$.

Results

Short-term epileptiform activity induced by kainic acid results in DNA methylation changes and altered mRNA level in GluR2 in in-vitro mouse hippocampal cultures

Initially, we were interested in the early phase of epilepsy as we wanted to know if DNA methylation was involved in the initiation of the disorder. We were able to create an experimental condition which mimics the initial stage of epileptogenesis with a short-term treatment of kainic acid (KA) to mature organotypic hippocampal cultures. At this stage, the neuronal network has just received an insult in the form of epileptiform activity and is ready to undergo network reorganization. We examined whether initial states of epileptogenesis are associated with changes in DNA methylation of the *GRIA2* receptor. Through bisulfate-pyro sequencing, we examined up to 800bp upstream of the *GRIA2* transcription start site where the promoter is located (Figure 3). The area of the promoter is homologous to the rat promoter characterized in an article by Myers *et al* [105]. There are a total of 40 CpG sites in this region (Figure 3). We found that the methylation levels are relatively low in this region (below 7%, refer to Figure 4a) and that the CpG sites in this region generally became hypomethylated in response to epileptiform activity (Figure 4a, *$p<0.05$, **$p<0.01$, ***$p<0.001$). The low level of DNA methylation in this area is expected since GluR2 is widely distributed in pyramidal cells in hippocampus. Also, the low level of DNA methylation in this area suggests that it is unlikely to have a major effect on the level of transcription. CpG Sites 33, 37 and 38 were not examined due to difficulties in assay design as a result of highly unfavorable DNA sequence in the region. However, there is an area starting 511bp (around CpG site 34) upstream of

the TSS that has a significantly higher methylation level (refer to Figure 4a) and an overall increase in DNA methylation was observed Site 34 – Control: 7.71±0.13%; KA: 9.43±0.41% (mean ± SD), $p<0.001$. Site 35 – Control: 8.99±0.07%; KA: 10.24±0.23% (mean ± STD), $p<0.001$. Site 36 – Control: 11.23±0.19%; KA: 12.45±0.41% (mean ± STD), $p<0.001$. Site 38 – Control: 15.98±0.20%; KA: 13.46±0.95% (mean ± STD), $p<0.001$. Site 39: 11.47±0.27%; KA: 13.46±0.64% (mean ± STD), $p<0.001$. Site 40 – Control: 19.12±0.17%; KA: 19.93±0.77% (mean ± STD), $p>0.05$. Interestingly, within proximity of this area, there contains a binding site for P300 (predicted by TFSearch http://www.cbrc.jp/research/db/TFSEARCH.html) which mediates the activities of a large number of transcription factors. Therefore, this area is more likely to have a greater effect on *GRIA2* transcription. The induction of epileptiform activity also significantly lowered GluR2 mRNA level compared to control (Figure 4B). Control: 0.8836±0.032, KA: 0.5247±0.0327 (mean ± SD), $p = 0.0001$. GluR2 mRNA levels were normalized to GAPDH mRNA levels. A reduction observed in GluR2 mRNA level correlated with DNA hypermethylation of CpG site 34 to CpG 40.

DNA methylation changes validated in a homologous region of GluR2 in an in-vivo rat model and correlated to seizure severity

To validate if *GRIA2* hypermethylation is sustained chronically *a*s well as to investigate any correlation between molecular changes and physiological outcomes, we examined an *in vivo* model of epilepsy. In brief, adult rats 9-10 weeks old were injected with kainic acid to induce status epilepticus. They were monitored with video-EEG recording for a period of 2 weeks for seizure

activities at 10 weeks post-kainic acid injection with surgical-implanted electrodes. Then they were sacrificed and Hippocampi were studied. An example of such electrographic seizure experienced by a rat is shown in Figure 5. Initially, we separated the rats into 2 groups according to the numbers of their seizures. By plotting the number of seizures for each rat, two distinct groups can be visualized (Figure 7); high convulsive rats (HKA, n = 4) and low convulsive rats (LKA, n = 9). A region in the rat *GRIA2* promoter which is homologous to the region which showed significant higher methylation and became hypermethylated in mouse was assessed. This area also contains a binding site for P300 similar to its mouse homology. Bisulfite-pyro sequencing was also implemented. There are 5 CpG sites in this region (Figure 6). The HKA group shows significantly higher DNA methylation compared to LKA and Control in all sites by 1-way ANOVA (Figure 8). Site 1 – Control: 33.48± 0.89%, HKA: 44.63±4.249%, LKA: 34.44±1.80% (mean ± SEM); $F(2, 20) = 7.343$, $p = 0.0041$. Site 2 – Control: 30.98±0.81%, HKA: 43.27±3.73%, LKA: 32.16±1.29% (mean ± SEM); $F(2, 20) = 13.33$, $p = 0.0002$. Site 3 – Control: 26.94±1.07%, HKA: 35.31±3.115%, LKA: 27.28±1.02% (mean ± SEM); $F(2, 20) = 7.594$, $p = 0.0035$. Site 4 – Control: 26.35±1.23%, HKA: 34.71±3.65%, LKA: 28.50±1.66% (mean ± SEM); $F(2, 20) = 4.054$, $p = 0.0333$. Site 5 – Control: 36.34±1.13%, HKA: 48.10±4.50% (mean ± SEM); $F(2, 20) = 7.556$, $p = 0.0036$. This shows that the level of DNA methylation at this locus is proportional to the severity of epilepsy. When the average DNA methylation across the 5 CpG sites is plotted against the numbers of seizures (There are 5 ranks of intensities, class IV and V, according to the Racine scale, which is frequently used to assess seizure intensities in animal studies), a linear trend can be

observed with a r value of 0.72 and the relationship is significant by Pearson's correlation ($p = 0.006$) (Figure 9). Our data show that the level of DNA methylation at this locus is correlated with the severity of epilepsy as well as the number of high convulsive seizures of an animal.

Discussion

Epilepsy is a neurological disorder that has yet to find a cure and the underlying mechanisms are not well understood. In this study, we used models of temporal lobe epilepsy to try to understand the progression and the late onset of the disorder, which is characterized by a time of chronic “silent period” between the initial injury and beginning of spontaneous seizures [106, 107]. Recently, one of the mechanisms that has been gaining interest with respect to gene regulation and long-term signature, is epigenetic [108]. Very limited experimental evidence on epigenetic mechanisms in epilepsy is available to date. Most of the published work focused on histone modifications. For instance, *GRIA2* which has been shown to play a role in epilepsy [14, 15] was also shown to be regulated by histone modifications [13]. However, the role of DNA methylation in epilepsy was unknown.

We used kainic acid to induce epileptiform burst in mouse hippocampal cultures *in vitro*. Then we measured DNA methylation levels at specific CG sites at the *GRIA2* promoter. We discovered low level of DNA methylation within close proximity of the transcription start site (TSS) with an increasing trend further upstream of the TSS. Significant increases in DNA methylation were detected at an area which is near a binding site for transcription factor p300. It suggests that this area can be a potentially important regulatory region. The increased methylation is anticipated to result in gene silencing. We therefore checked whether mRNA level of *GRIA2* was down-regulated. We demonstrate that *GRIA2* downregulation was correlated with an increase in DNA methylation.

The down-regulation of *GRIA2* agrees with published results from different studies of epilepsy [14, 15]. The changes in DNA methylation happened rapidly after a 2-hour period. This suggests that the methylome is highly dynamic and is able to respond to environment stimuli very rapidly, which is consistent with the notion that the neuronal network must be highly plastic to be able to carry out its functions in cognition and behavior. The 2-hour duration was chosen because we wanted to avoid hippocampal lesion (namely loss of CA3 pyramidal neurons) [109] in order to examine the early phase of the disorder. In our *in vivo* model, we saw a chronic increase in DNA methylation n *GRIA2* which is homologous to the one examined in the *in vitro* model. This evidence is consistent with the hypothesis that there are chronic alterations in the methylome that are triggered by the initial insult and remain stable over a period of time. We suspect that this is a result of neuronal network's response in an effort to try to recover from seizure (or epileptiform activity)-induced damage through chronic neuronal network reorganization events. From both *in vivo* and *in vitro* data, it seems that the examined region in *GRIA2* promoter may be an important regulatory region. Future experiments can be done to test how DNA methylation of this region can influence *GRIA2* transcription. One can carry out the experiment by first cloning the region and methylating it *in vitro.* Then the methylated piece can be inserted into a vector containing a reporter. Afterwards the engineered plasmid can be transfected into cells (preferably of neuronal origin) and reporter expression can be compared between methylated and unmethylated conditions. In addition, GluR2 protein level should be checked as mRNA levels do not necessarily translate to protein levels.

Further, it is important to compare GluR2 localization in the hippocampus cell types.

The level of chronic increase in *GRIA2* DNA methylation is positively correlated with the numbers of recorded seizures. This implies that DNA methylation may play a role in determining the severity of the epilepsy by programming the genome of a cell to respond in a certain way within a neuronal network. The positive correlation observed between *GRIA2* methylation and severity of epilepsy is inconsistent with the idea that the extent of silencing of *GRIA2* defines the severity of epilepsy. Reduced GluR2 would lead to increased excitability of neurons and lower seizure threshold. In order to test this, future experiments will need to address how different levels of DNA methylation can influence kainic acid-induced epileptiform activities.

Key epigenetic regulators such as DNMT1, DNMT3a, MBD2, and others can be knocked down to decipher the underlying mechanisms. Drugs that interfere with epigenetic proteins can also be used to study the mechanism involved: 5-azacytidine (an inhibitor of DNMTs [110]), RG108 (an inhibitor of DNA methyltransferases [111]), SAM (S-Adenosyl methionine, the methyl donor for DNA methylation reaction. Increasing intracellular level of SAM leads to increased global methylation level [112]) and SAH (S-Adenosyl-L-homocysteine, a metabolite of SAM, increasing intracellular level of SAH inhibits activities of DNA methyltransferases[113]). Once the methylome has been disrupted either with RNAi or with drugs, the effect of these disturbances on the induction of epileptiform activity can be examined. *In vivo* experiments can be repeated to include these elements to further investigate the impact of *GRIA2* DNA methylation level on the severity of

seizures.

Our *in vivo* model showed a range of responses to kainic acid in terms of *GRIA2* DNA methylation levels and number of seizures. This is a very remarkable finding as it implies that though the rats are inbred and supposedly identical, there are still subtle differences in their epigenome possibly due to slight variations in pre- and post-natal experience that resulted in differential phenotypic and epigenetic responses to stimuli. This observation is applicable to human populations because not everyone who has experienced brain insults will develop epilepsy; for example, one study has reported 20 out of 1000 children who has experienced febrile seizures have developed epilepsy seven years after [114].

The changes in *GRIA2* DNA methylation in the *in vitro* culture is small and it does not seem to match an almost 50% change in mRNA level. A possible explanation for this discrepancy is the fact that we are using whole hippocampi which are mixes of various populations of cell types and GluR2 is only expressed in neurons [115]. DNA methylation is like a digital signal; a CpG site at a given cell is either methylated or non-methylated. Percentages of DNA methylation that we measured basically mean that a certain fraction of the whole population of cells have a methylated cytosine at that CpG site. Therefore, by using a whole hippocampus, the effect may have been diluted by non-neuronal cells. In addition, hippocampal cultures from different mice were pooled in an attempt to increase DNA and RNA yield. Pooling of samples were not previously thought as a problem until we examined the data from kainic acid-treated rats where we saw individual

variations in response to seizure, both molecularly (different *GRIA2* methylation levels) and physiologically (different severities of epilepsy). Therefore, the true effects may have been masked by the above reasons. For future experiments, it will be very important to study pure populations of cell types and examine every animal individually. Antibodies against NeuN (a nuclear protein, which is specific to neurons) can be used to separate out neuronal nuclei. Then DNA and RNA can be extracted. Genome-wide mRNA and DNA methylation analyses can also be used to pinpoint novel targets for future studies and possible therapeutics.

It is possible that changes in rat *GRIA2* DNA methylation is a result of granule cell proliferation according to a study by Jessberger et al. (2007) [81]. This is not consistent with our experiment since we did not detect significant cell division by BrdU incorporation in our cultures (data not shown). In their study, they induced SE in rats with kainic acid and saw that valporic acid (an anti-seizure drug and also a histone deacetylase inhibitor) was able to abolish seizure-induced granule cell proliferation but not neurodegeneration. In addition, they showed that by treating the rats with valporic acid after the onset of SE, GluR2 down-regulation was prevented. Although their report certainly established a link between neurogenesis and GluR2 down-regulation as a result of HDAC activity, it is still uncertain that the epigenetic changes observed in both studies solely occurred in newborn granule cells. Future *in vivo* experiments can include using a combination of BrdU administration and laser capture microdissection to examine if seizure-induced epigenetic changes happen only in newborn granule cells. Further, it is possible that histone acetylation plays a

big role in regulating *GRIA2* expression level. In fact it is expected that differences in histone acetylation will be detected in our experimental models as DNA methylation and histone modifications are so closely related, such that histone acetylation can promote replication-independent DNA demethylation. Regardless, histone acetylation in the *GRIA2* locus should be examined in order to achieve a better understanding of how epigenetic mechanisms control the expression of this gene.

Our experimental data shows that a short epileptiform activity is sufficient to produce rapid changes in DNA methylation of *GRIA2* (and most likely in other loci as well) even in post-mitotic cells (no significant detection of cell division in our *in vitro* cultures by BrdU incorporation [data not shown]). Although the precise mechanism remains unknown, we suspect that the DNA methylation machineries that are important in normal brain physiology are affected by the insult. This then causes deregulation in DNA methylation and result in alterations in the long-term programming of gene expression that can drive network reorganization events. Over time, a neuronal network capable of generating spontaneous seizures is formed.

Conclusion

Our results provided a novel extension to the classic understanding of epileptogenesis. We show that induction of an epileptic seizure causes a sustainable methylation of a specific area of the *GRIA2* promoter. This methylation change is highly correlated with the expression of GluR2 and the development of epileptogenesis. The sequence specificity of the methylation changes suggests a regulatory mechanism which might be a new therapeutic target.

The characterization of *GRIA2* serves as a starting point for future investigations into the aberrant epigenetic regulations in epileptogenesis. Our data suggest that there are rapid DNA methylation changes in response to epileptiform activity. These changes persist long after the epileptiform activity trigger is removed and they are positively correlated with severity of epilepsy. Interestingly, within the same litter-mate there are individual variations in the epigenetic changes which might explain the phenotypic differences in rats after kainic acid treatment. In addition to other variables such as genetic variation, epigenetic variation in human population may determine the susceptibility of individuals to epileptogenesis after the initial triggering event. Further investigation is required to unravel the precise relationship between aberrant epigenetic regulations and epileptogenesis. By understanding the epigenetic responses and mechanisms for epileptogenesis, new doors can be opened for intervention in epileptogenesis with novel therapeutics.

Figures

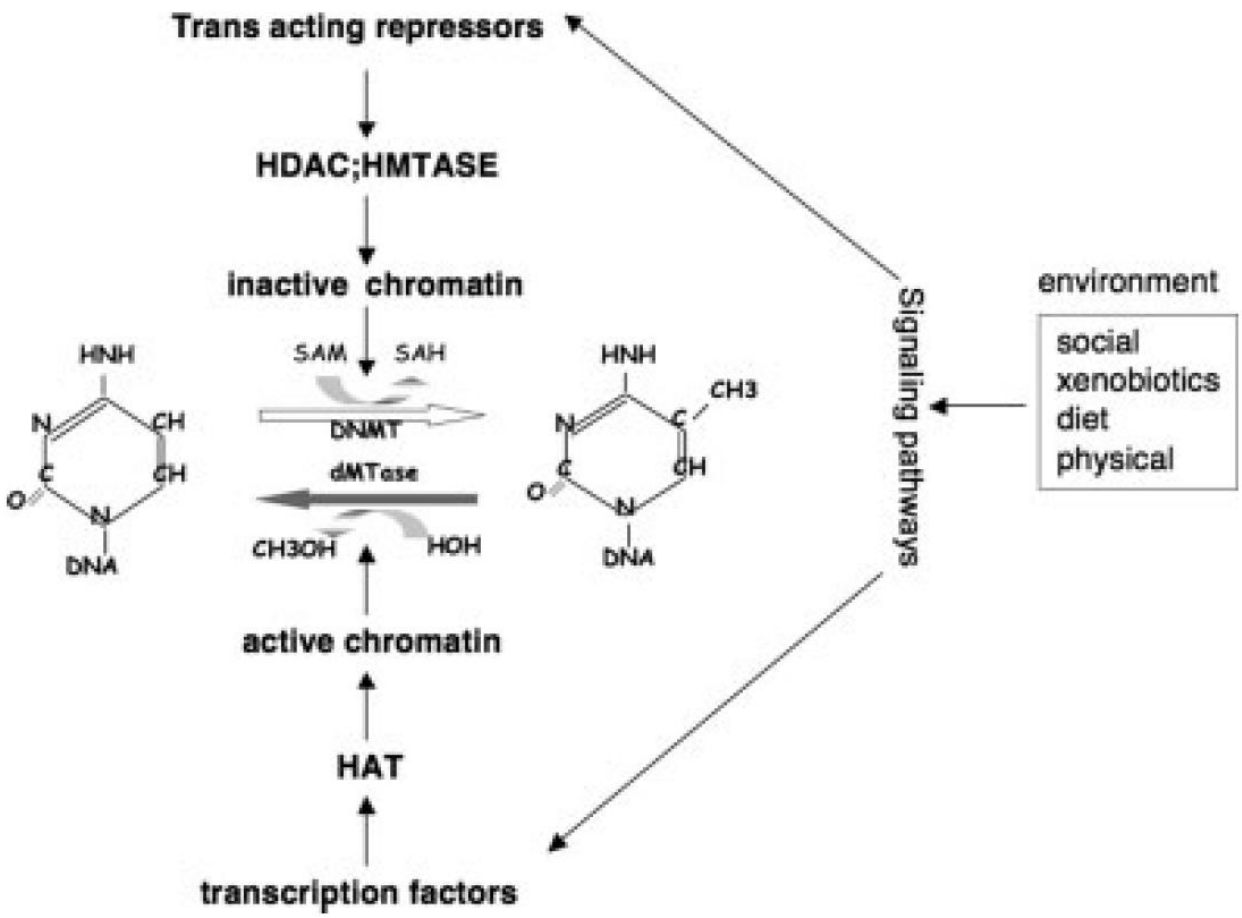


Figure 1. (Adapted from Szyf *et al*. 2008). The interaction between environment and the dynamic epigenome. The state of methylome is determined by the balance between methylation and demethylation reaction. Different environmental signals can elucidate various cellular signaling pathways to induce a variety of trans-acting elements with either activating or repressing properties. These trans-acting elements can recruit histone-modifying enzymes to help modify chromatin states. Active chromatin state promotes DNA demethylation where inactive chromatin state promotes DNA methylation.

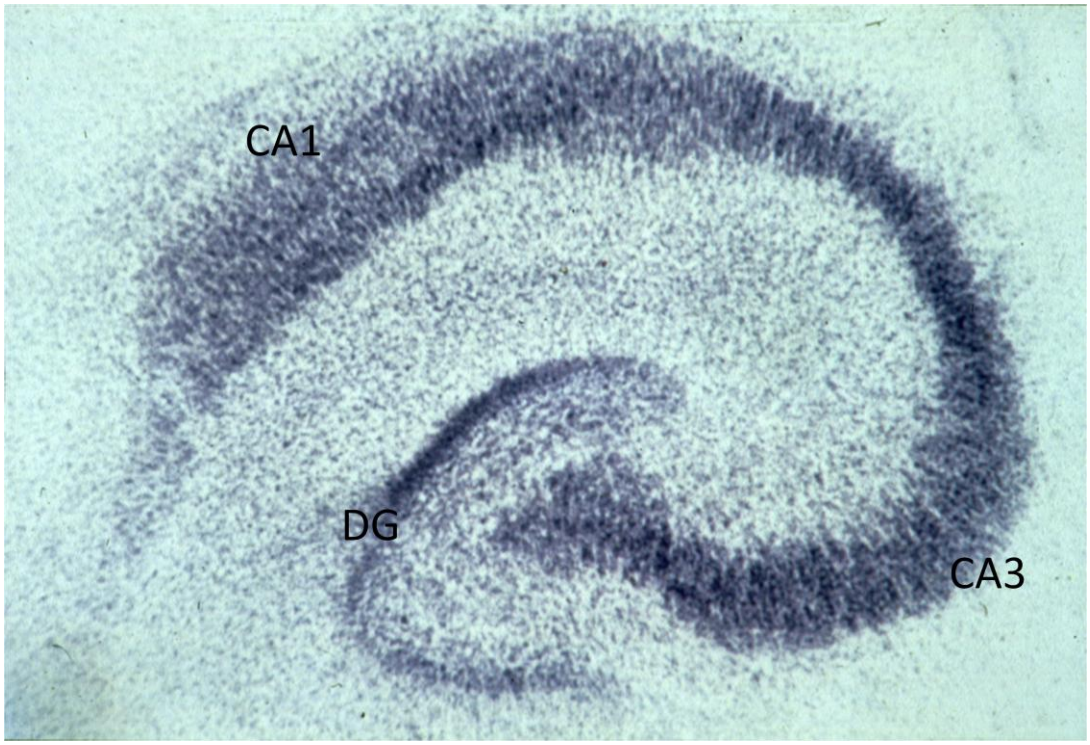
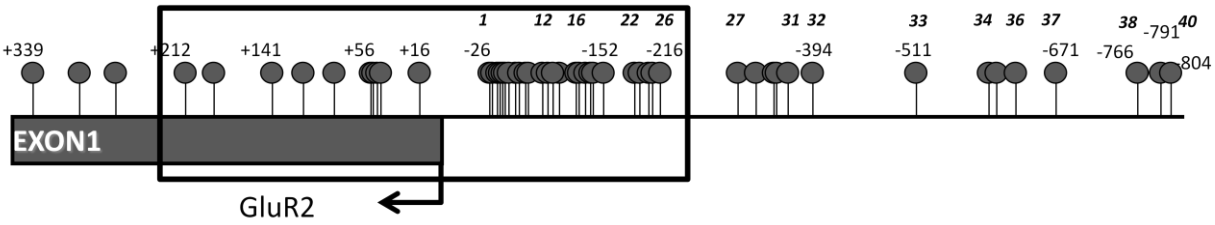
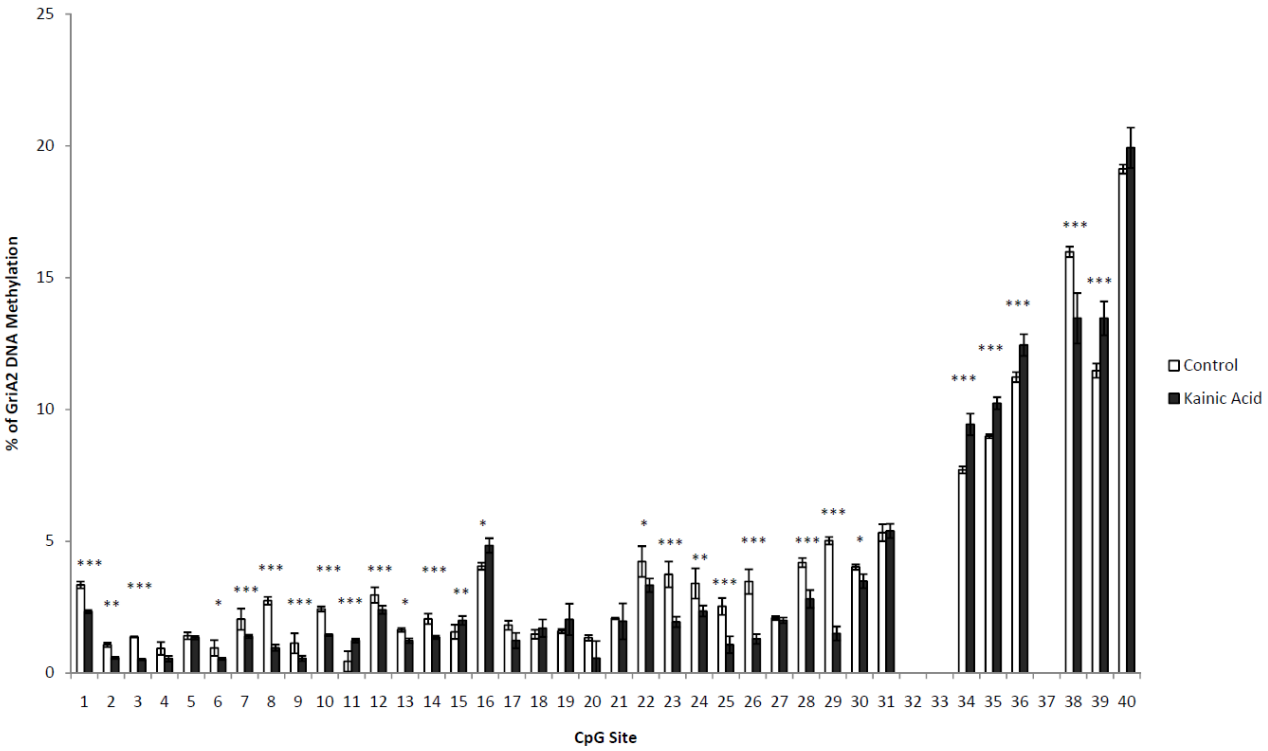


Figure 2. A Nissl stained mouse organotypic culture at 3 weeks of age. Note the cytoarchitecture of the hippocampus is maintained.

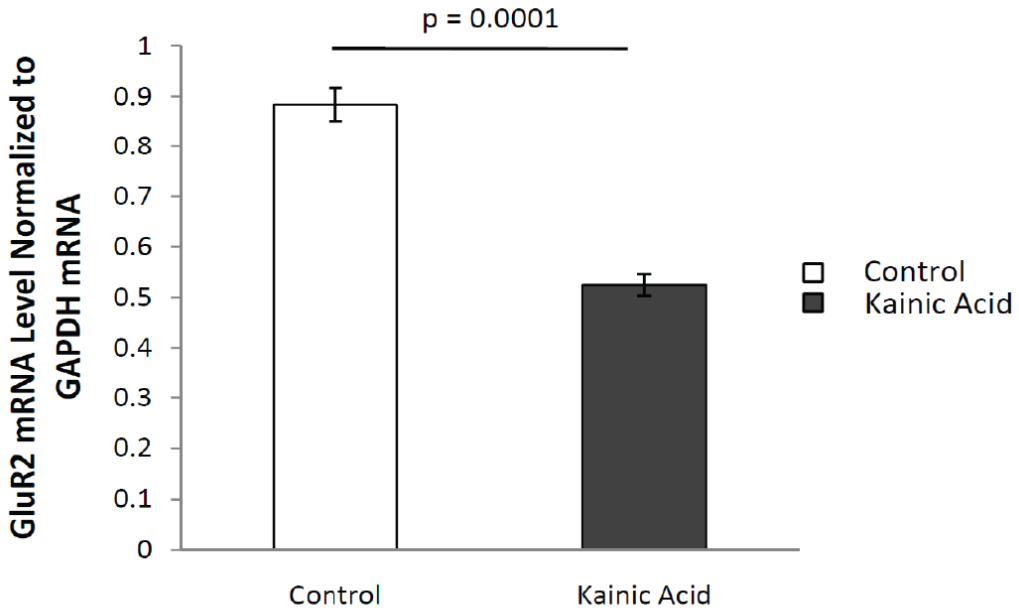


<u>Figure 3.</u> A schematic diagram showing the map of the mouse *GRIA2* promoter region. Boxed area represents the actual promoter area. All the CpG sites upstream of the transcription start site in this diagram were assessed by bisulfate pyro-sequencing. Each lollypop represents a CpG site. There are a total of 40 CpG sites in the area upstream of transcription start site (TSS) in this diagram that were examined.

A)



B)



<u>Figure 4.</u> DNA methylation at *GRIA2* gene and gene expression levels on mouse organotypic cultures after experiencing epileptiform activities. A) Assessment of DNA methylation level at the promoter region of moue *GRIA2* by bisulfite-pyrosequencing revealed changes in CpG methylation

at this locus. DNA methylation levels are relatively low (less than 7%) before CpG site 34 and this may be unlikely to have a major impact on *GRIA2* gene regulation. A significant increase in DNA methylation level was observed upstream to site 34. Furthermore, average methylation level is increased from site 34 to site 40 (*p<0.05, **p<0.01, ***p<0.001). The higher methylation level and higher differences after KA treatment suggest that this region may have additional roles in *GRIA2* regulation. **B)** GluR2 gene expression quantified by qRT-PCR. GluR2 mRNA level was normalized to GAPDH. The analysis was done in triplicates. The results revealed a significant down-regulation of GluR2 after treatment. This correlates with an increase in DNA methylation of the gene.

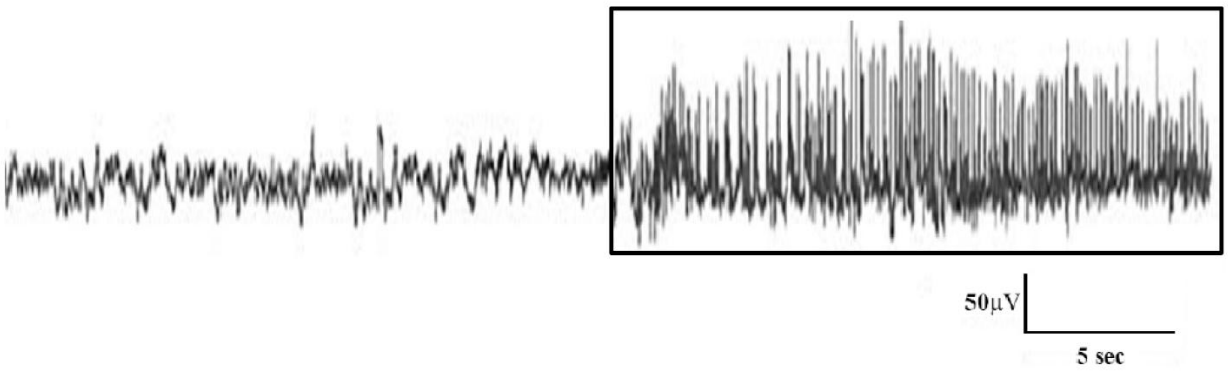


Figure 5. An example of electrographic illustration of a seizure from one of the rats. A typical epileptic discharge is highlighted in the box. The data was recorded with accompanying synchronous video using Compumedics software.

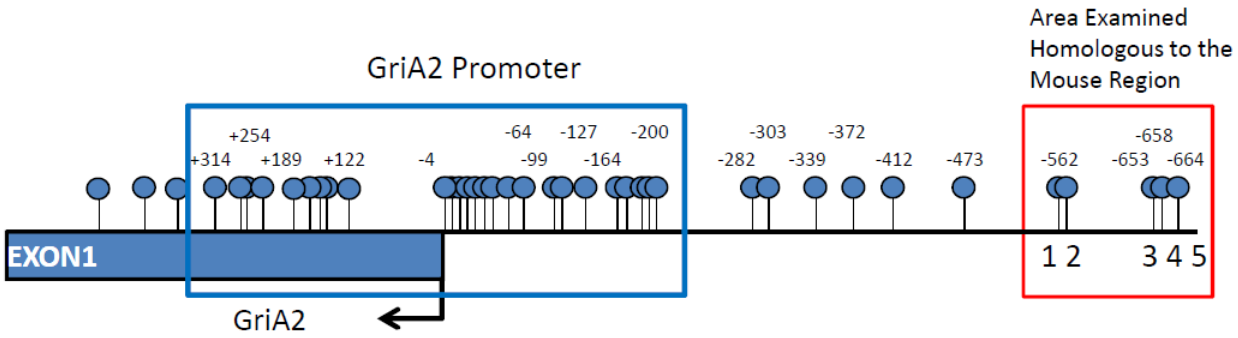


Figure 6. A schematic illustration of rat *GRIA2* promoter region. Boxed areas indicate the minimal rat *GRIA2* promoter and the examined region which is homologous to the one in mouse. Each grey lollypop represents a single CpG site and there are 5 in the area examined.

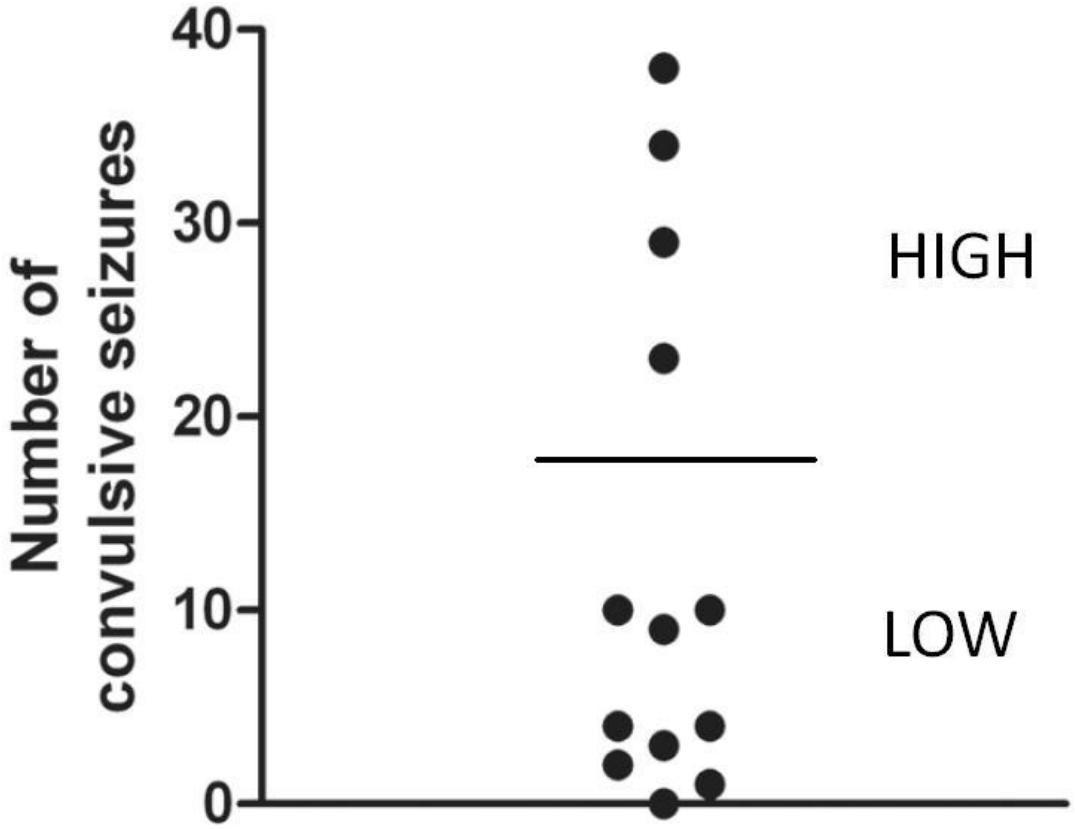


Figure 7. Inter-individual variations in the number of seizures. The numbers of seizures (Class IV and V) that were recorded during the two weeks of video-EEG session in KA-treated rats. Two distinct groups can be identified with their seizure profiles. Rats in the higher group were assigned as high-seizing whereas the ones in the lower group were assigned low-seizing.

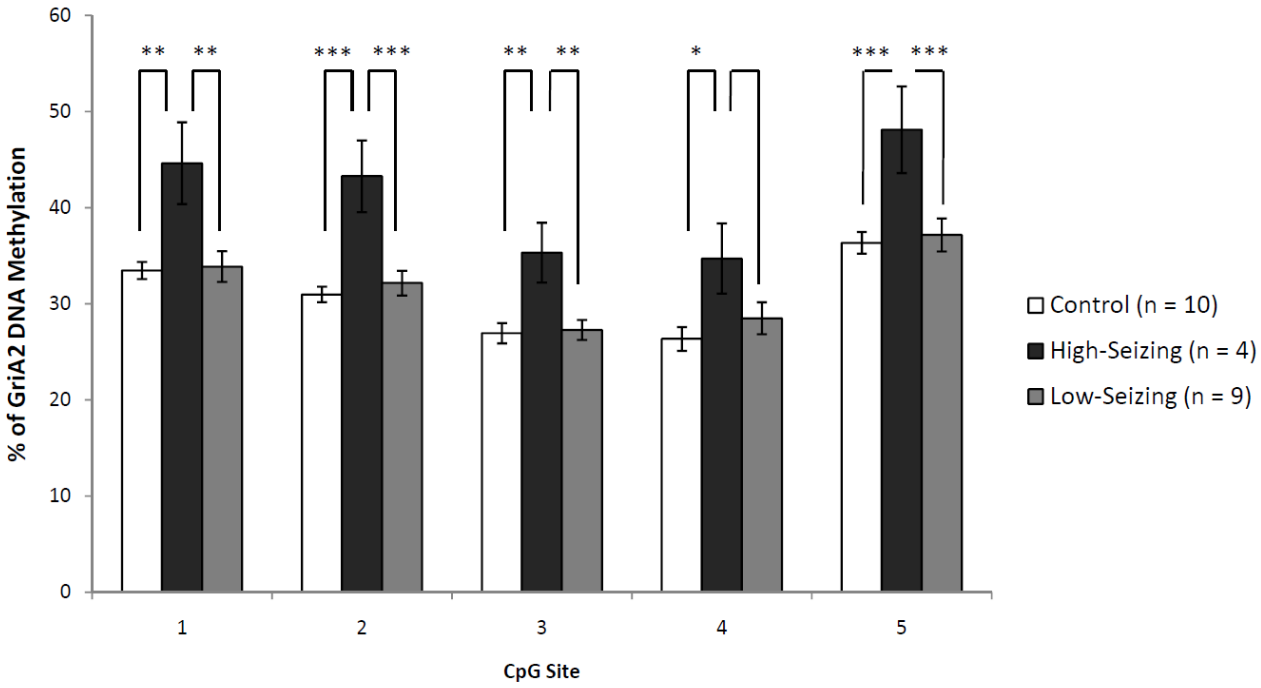


Figure 8. Comparison of DNA methylation levels at rat *GRIA2*. This region is homologous to the area examined in mouse. High-seizing rats display significant higher methylation at all five CpG sites comparing to Low-seizing and Control rats. No significant differences were observed between Low-seizing and Control rats. (*p<0.05; **p<0.01; ***p<0.0001). This implies that the level of *GRIA2* DNA methylation is correlated with the severity of epilepsy.

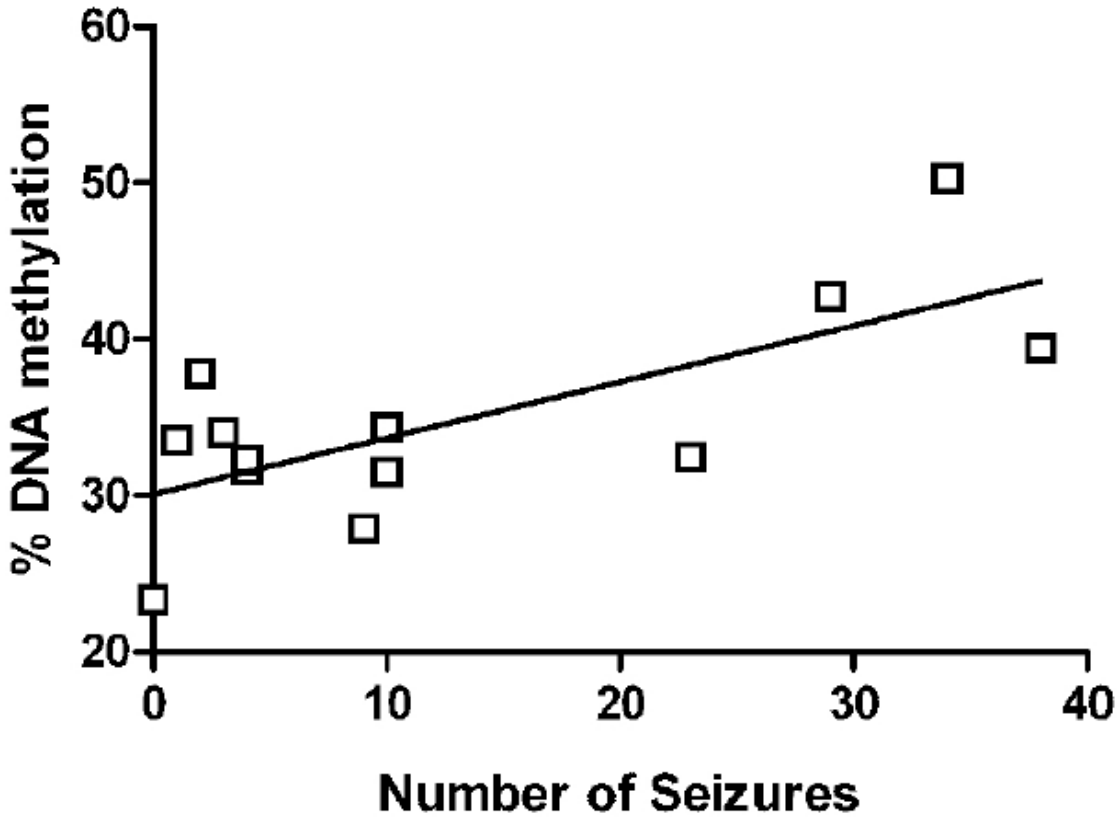


Figure 9. Correlation between *GRIA2* methylation and number of seizures. By plotting the average % of methylation across the 5 CpG sites in rat *GRIA2* versus numbers of seizures (class IV and V, on Racine scale), a significant correlation can be revealed ($r=0.72$; $p=0.006$). Each square represents an individual rat. This shows that there is a relationship between frequency of seizure generation and GluR2 DNA methylation.

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