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NON-CONVULSIVE STATUS EPILEPTICUS IN A PERIADOLESCENT RAT MODEL: EVIDENCE FOR ALTERATIONS IN HIPPOCAMPAL SYNAPTIC PLASTICITY AND LATER LIFE DISTURBANCES IN COGNITIVE AND EMOTIONAL BEHAVIORS

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
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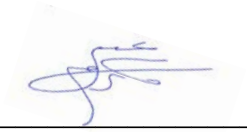

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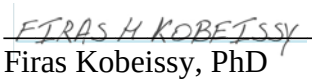

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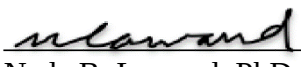

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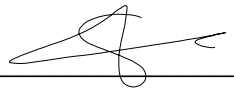

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ABSTRACT
OF THE THESIS OF

Sana M.Amin Alturk for Master of Science
Major: Neuroscience

Title: Non-Convulsive Status Epilepticus in a periadolescent rat model: evidence for alterations in hippocampal synaptic plasticity and later life disturbances in cognitive and emotional behaviors.

Background: Non-convulsive status epilepticus (NCSE) refer to prolonged seizures with minimal motor involvement. In clinical practice, these seizures are often underdiagnosed due to subtle manifestations such as transient behavioral changes. While convulsive status epilepticus (tonic-clonic motor seizures) is universally aggressively treated within minutes to prevent its indisputable harmful consequences and ensuing brain damage, NCSE management remains controversial and often not aggressive over hours to days as its consequences on the brain remain elusive. Pilot experiments in our laboratory, in line with few recent reports, have revealed possible early learning deficits days following NCSE in our recently established periadolescent rodent model. Here, we aim at confirming these early deficits in emotionally-relevant learning following one or two NCSE episodes given that this condition often recurs prior to coming to medical attention. Furthermore, we examine possible electrophysiological, structural, and molecular underlying mechanisms for such deficits. Additionally, we investigate whether emotional and cognitive behavioral deficits are present one month following NCSE.

Methods: Male postnatal day 43 (P43) peri-adolescent rats received one (SKA group), or two 24 hours apart (RKA group), intra-hippocampal 0.00625μg injections of kainic acid to induce prolonged non-convulsive seizures (NCSE) under continuous EEG monitoring. Controls were sham treated with normal saline. In the short-term experimental paradigm, animals were subjected to the modified active-avoidance (MAAV) test at P45, then sacrificed to perform immunohistochemistry for neuronal nuclear antigen (NeuN), glial fibrillary acidic protein (GFAP), and the synaptic plasticity marker, synaptophysin (Syp). In the long-term paradigm: animals were subjected to continuous EEG monitoring followed by a battery of behavioral tests one month post-NCSE. The behavioral panel included tests for anxiety-like behaviors (light-dark box (LD) and open field (OF) tests), depressive-like behaviors (forced swim test (FST)), and hippocampal-dependent visuospatial navigation (Morris water maze (MWM)), followed by the MAAV test.

Results: All rats developed one (SKA) or two (RKA) episodes of hippocampal NCSE following KA injections, with electrographic patterns of evolving rhythmic fast spikes and polyspikes, accompanied by behavioral arrest, staring, and oromotor automatisms.

Average post-KA latencies to NCSE were comparable between SKA and RKA on the first day of induction, as were the average seizure durations. On the second day of induction, RKA rats had comparable latencies and durations to the first day of induction. In the short-term paradigm, daily acquisition of tone-signaled electrical foot-shock avoiding behaviors in the MAAV revealed a statistically significant ($p < 0.05$) deficit in avoidance rates of both the SKA and RKA groups when compared to controls. In the auditory retention subset, while SKA rats had avoidance rates comparable to controls, RKA rats exhibited statistically significant ($p < 0.05$) lower retention rates. SKA and control groups were significantly ($p < 0.05$) faster in acquiring adaptive context-cued shock avoidance ($p < 0.05$) when compared to the RKA group that also exhibited deficits in contextual retention ($p < 0.05$). No overt cell loss was observed in hippocampal NeuN stained sections. However, compared to controls, both the SKA and RKA groups had a statistically significant increase in reactive astrogliosis in both the left and right hippocampal hilar regions, and CA2-CA3 regions ($p < 0.05$). RKA rats had significantly decreased hippocampal Syp levels in the hilar and CA2-CA3 regions ($p < 0.05$) when compared to both SKA and control groups which were comparable. In the long-term paradigm, continuous EEG recordings revealed no seizure recurrence but an increase in spike and polyspike frequencies in both the SKA and RKA groups compared to controls. One month post-NCSE, the RKA rats had deficits in visuospatial navigation in the MWM and in contextual learning in the MAAV test compared to both SKA and control rats. The LD and OF tests pointed to potential hyperactivity and anxiety-like behaviors with decreased exploratory tendencies in the RKA group. Both the SKA and RKA groups showed increased immobility in the FST, suggestive of possible depressive-like behaviors.

Conclusions: Here we confirmed the presence of early behavioral deficits following NCSE, and our pilot data points to the persistence of such deficits along with depressive and anxiety-like behaviors one month following two, but not one, episodes of NCSE. These deficits were accompanied by hippocampal injury evidenced by reactive astrocytosis and alteration in synaptic homeostasis. Ongoing studies in our laboratory aim at confirming the persistence of these molecular changes and detrimental behavioral deficits. These harmful consequences, and the impact of seizure burden, if confirmed, call for a more urgent diagnosis and treatment of NCSE.

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CA	Cornu Ammonis
CSE	Convulsive Status Epilepticus
CTR	Control
DG	Dentate Gyrus
EEG	Electroencephalography
FST	Forced Swim test
GABA	Gamma-Aminobutyric acid
GFAP	Glial Fibrillary Acidic Protein
KA	Kainic Acid
KAR	Kainate receptor
LDT	Light-Dark Box Test
LTD	Long-term depression
LTP	Long-term potentiation
MAAV	Modified Active Avoidance
MWM	Morris Water Maze
NCSE	Non-Convulsive Status Epilepticus
NMDA	N-Methyl-D-aspartic acid
OFT	Open Field Test
P	Postnatal day
PFA	Paraformaldehyde
RKA	Repeated Kainic Acid

SE	Status Epilepticus
SKA	Single Kainic Acid
SRS	Spontaneous recurrent seizure
SUDEP	Sudden Unexpected Death in Epilepsy
Syp	Synaptophysin
TLE	Temporal Lobe Epilepsy

CHAPTER I

INTRODUCTION

The nervous system affects nearly every aspect of our bodily functions, health, and well-being. It guides all our actions and controls complex as well as simple processes in our bodies. This system is made up of billions of neuronal cells forming a network that is delicately maintained in a state of electrophysiological balance to ensure proper functioning. In the face of developmental and environmental changes, our central nervous system acts to restore and sustain the balance, however, whenever it fails, disorders may arise.

A seizure provides one of the clearest examples of a neuropathological state triggered by a disrupted electrophysiological balance. It consists of abnormal excessive, or synchronous neuronal discharges, primarily in the cerebral cortex, manifesting clinically with sudden motor, sensory, or behavioral changes with or without various levels of alterations in consciousness (1). In the most dramatic form, it may cause uncontrollable rhythmic- clonic or tonic muscle stiffening, known as convulsions. However, seizures may also be clinically subtle and go nearly unnoticed. While seizures may be provoked by an acute systemic illness or brain insult (trauma, infection, toxic exposure, or high fever); some individuals have an enduring predisposition to recurrent unprovoked seizures as a result of a genetic or acquired epileptogenic network (2, 3). These individuals are considered to have “Epilepsy”; a chronic neurological disorder characterized by recurrent unprovoked seizure activity. Epilepsy has deleterious functional, and potentially structural effects on the brain leading to substantial morbidity and mortality. These effects may be worsened by prolonged convulsive

seizure activity, frequently referred to as “Convulsive Status Epilepticus” (CSE), which is a life-threatening medical emergency. Damage caused by such seizures is dependent on brain structures from which they originate and network connections between these structures, an example being temporal lobe epilepsy (TLE); the most common form of localization-related (focal) epilepsies that is often refractory to anti-seizure medications (ASMs) (4). TLE seizures mainly involve the amygdalar nuclear complex and the hippocampal formation and their interconnections. These structures are key components of the limbic system having a critical role in emotion, cognition, and complex executive functions (5). In rodents, dysfunction of these structures and their circuitry leads to deficits in learning, memory, spatial navigation, and emotional fear conditioning (5, 6). Seizures arising from the temporal lobe can spread to involve both sides of the brain causing tonic-clonic convulsions, or they may remain localized with non-convulsive manifestations. Non-convulsive seizures refer to seizures that lack tonic muscle stiffening or clonic rhythmic muscle jerking. In TLE , non-convulsive seizures clinically manifest with behavioral arrest, amnesia, hallucinations, paroxysmal language difficulties, and repetitive purposeless movements termed automatisms (7). Unlike convulsive seizures, these are difficult to detect, and thus are frequently missed and undertreated, or come late to medical attention following multiple recurrences. Several reports have pointed to potentially harmful consequences of prolonged non-convulsive seizures, termed Non-Convulsive Status Epilepticus (NCSE), although research data on this subject remains scarce. While brain damage is shown to occur following CSE (8), the potential harmful effects of NCSE on the brain therefore remain elusive. Unlike convulsive SE which is treated aggressively within minutes with escalation to anesthetic

drugs if needed in order to avoid neuronal loss, there are no well-established guidelines for the treatment of NCSE which is often treated over hours to days (9) .

In biomedical research, animal models have been developed to investigate the underlying mechanisms of disease processes and to discover novel methods for prevention, diagnosis, and treatment. Rodents have been the most widely used animal species in neuroscience research for well over a century. Experimental models of prolonged seizures or SE are frequently produced using pharmacologic agents or by electrical kindling to mimic seizure representation in humans. In our laboratory, a novel peri-adolescent rodent model of temporal lobe NCSE has been established using intra-hippocampal Kainic Acid (KA) as a proconvulsent to investigate potential behavioral and molecular alterations associated with this condition. In this study we aimed at exploring acute molecular underpinnings of hippocampal NCSE. Additionally, we examine potential later life effects of NCSE on amygdalo-hippocampal-related functions, namely cognitive and emotional behaviors, learning, memory, spatial navigation, adaptability, and depressive and anxiety-like behaviors.

Below is a review of seizure characteristics and their effects on brain functions with a focus on those originating in the temporal lobe, notably temporal lobe epilepsy, preceded by a short overview of neuronal homeostatic regulation mechanisms responsible for maintaining network balance. We will then provide a brief description of the amygdalo-hippocampal circuitry and its involvement in cognitive and emotional behaviors. Afterwards, we will review NCSE; shedding light on most recent reports discussing its consequences on functional and molecular integrity of brain structures. Then we will review the most common chemoconvulsant seizure model used to study

this condition in support of employing our recently established hippocampal NCSE model to investigate long-term effects.

A. Homeostatic regulation of neuronal function

All living organisms exhibit the ability to maintain balance in their physiological internal environment (‘milieu interior’) despite external changes, a biological concept known as Homeostasis. In the human body, multiple systems often work together to sustain its balance, and if they fail, a disease or even death might be the result. For example, important physiological factors such as oxygen levels, blood pH, electrolyte concentrations, and core body temperature all are maintained within an acceptable range of values. The nervous system is usually responsible for detecting internal and external distress signals and coordinating appropriate homeostatic responses in addition to its highly complex ability to anticipate potential disruptions (10). At the same time, the nervous system itself is subject to continual disturbances due to environmental fluctuations and developmental changes. On a cellular level, neuronal homeostatic processes were also observed in different experimental preparations and contexts. Neurons can alter their intrinsic excitability or synaptic strength to ensure an appropriate electrophysiological function (10). This includes alterations in ion channel densities as well as in the size or shape of pre-synaptic and post-synaptic structures in order to maintain a neuronal firing rate at an appropriate level. This activity-dependent regulation of intrinsic properties is often referred to as homeostatic plasticity (10).

1. Neuronal intrinsic plasticity

Controlling neuronal inward and outward membrane conductance is one of the mechanisms of maintaining cell-wide electrical homeostasis. It is dependent on the combined action of low-level regulatory mechanisms affecting the density of ion channels, the expression of their protein subunits, and their post-translational modifications and interactions. For example, an event of neuronal hyperexcitation, often detected by an increase in intracellular Ca^{+} concentration, can be compensated for by regulating the expression of hyperpolarizing K^{+} channels or passive leaky channels, through modifying transcription, trafficking, or translation of their constituents, in order to obtain an optimum level of activity (11, 12) (see figure 1). This phenomenon has been studied in primary hippocampal cultures as a model of homeostatic regulation of intrinsic excitability (11). In that model, chronic depolarization, achieved by applying high concentrations of extracellular KCl to culture media, induces a compensatory shift in the membrane excitability threshold (known as, rheobase current threshold), an increase in leak conductance, a decrease in input resistance, and repetitive spiking irregularity (11).

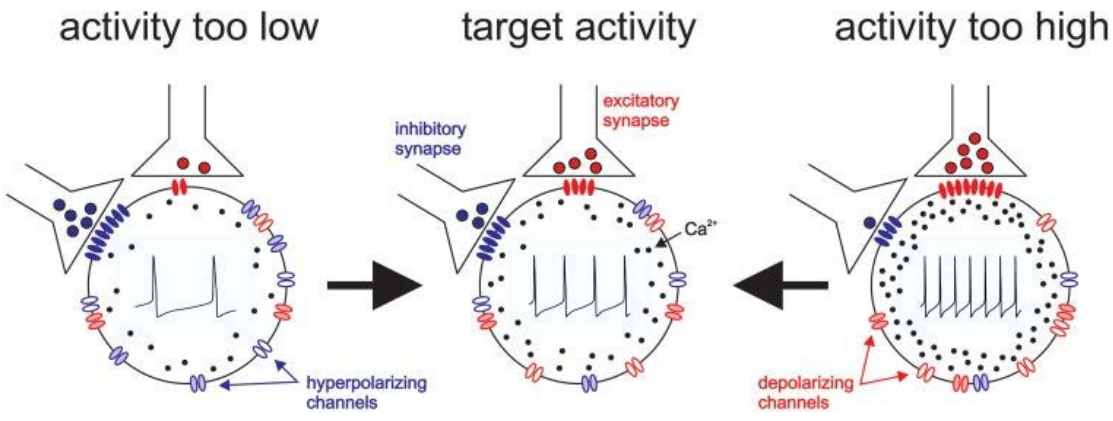

Figure 1 HOMEOSTATIC REGULATION OF NEURONAL FUNCTION
Illustrating activity-dependent regulation of neuronal intrinsic properties. In the case of low synaptic activity (left), intracellular Ca^{+2} concentration falls below its target value inducing increased recruitment

of depolarizing channels to enhance neuronal firing. In contrast, in a state of increased synaptic excitability (right), increased Ca^{+2} concentration results in an upregulation of hyperpolarizing channels and a downregulation of depolarizing channels to return the neuron's activity to its target level (13).

2. Neuronal synaptic plasticity

In the face of perturbations, compensatory changes in synaptic signaling are triggered to maintain neuronal excitability at a relatively stable level. It was observed that in response to developmental and learning-related changes, modifications of neuronal synaptic transmission can act to stabilize neuronal firing rate and prevent uncontrollable increasing or dying out (14). To illustrate, one of the best-understood forms of synaptic plasticity is synaptic scaling. This phenomenon has been tested, both in-vitro and in-vivo, on neocortical and hippocampal pyramidal neurons. It refers to the ability of individual neuronal cells to detect chronic changes in their own firing rate and respond accordingly by altering receptors accumulation at synaptic sites to maintain a stable function (10). For example, in a state of chronic inactivity and a decreased action potential firing (detected by Ca^{+} dependent sensors), scaling factors (such as brain-derived neurotrophic factor (BDNF) and cytokine tumor necrosis factor α (TNFα)) can act to upregulate surface α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) in order to increase neuronal post-synaptic response to the glutamatergic excitatory input which can be measured by recording miniature excitatory postsynaptic currents (mEPSCs) as a unit strength of a synapse (12, 15). Another phenomenon acting to control homeostasis of neuronal firing while maintaining synaptic efficacy is metaplasticity. It is defined as an activity-dependent and persistent modification of a neuron's future capacity for Hebbian plasticity (16-18). It tunes synapses' tendency to exhibit long-term potentiation (LTP) or long-term depression (LTD) by regulating the quantal release of neurotransmitters on both excitatory and

inhibitory neurons based on the overall network demands (16-18). The goal is to prepare neuronal networks for subsequent learning and long-lasting memory storage and to prevent maladaptive or pathological runaway potentiation or depression of synapses (19). In this way, it plays a defensive role against potentially noxious excitability increases by activating N-methyl-D-aspartate receptors (NMDARs) to suppress LTP and enhance LTD (19). However, these effects might restrain future learning that is dependent on LTP and LTD balance. Failure of metaplastic mechanisms in certain brain regions such as the hippocampus or amygdala may lead to impairments in cognition, learning and memory, and complex emotional behaviors (20). Thereby, these mechanisms may prove to be useful therapeutic targets in a variety of neurological disorders. Accumulating reports have been suggesting a role of disrupted metaplasticity in the development of cognitive impairments in Alzheimer's disease, Parkinson's disease, and Huntington's disease (21-24). For instance, a recent study has reported reestablishment of plasticity and associativity in hippocampal memory circuitry in an animal model of Alzheimer's disease by inducing metaplastic mechanisms (25). Several molecular factors are thought to be implicated in metaplastic processes, one of them is Synaptophysin (Syp).

3. Synaptophysin: a plasticity marker

Synaptophysin is the second most abundant molecule at synaptic regions of neurons (27). It is a calcium-binding integral membrane glycoprotein located to presynaptic small vesicles comprising ~10% of total synaptic vesicle protein content (26, 27). Most recent evidence demonstrates a key role of this molecule in modulating the efficiency of the synaptic vesicle cycle rather than directly influencing

neurotransmitter release (28). This is achieved by ensuring efficient trafficking and retrieval of synaptobrevin-II (SybII), which is essential for vesicles docking and fusion (exocytosis) to presynaptic membranes (28) (see figure 2). This role is most apparent in neurons or circuits undergoing periods of intense neuronal activity particularly during early brain development (28). Since Syp is present in virtually all neurons that participate in synaptic transmission, it is considered a specific and sensitive marker of synaptic terminals, thus immunostaining of Syp has been commonly utilized in the quantification of synaptic density (29). In rodents hippocampi, Syp reactivity is mostly apparent in layers lacking principal cell bodies and exhibiting a characteristic staining of neuropil (30). Syp expression has been shown to decrease in Alzheimers' patients hippocampi correlating with the severity of neuropathology and memory deficit (31). Moreover, increased exploratory behavior, impaired object novelty recognition, and reduced spatial learning have been observed in Syp knock-out mice (32). Additionally, several mutations within the Syp encoding genes have been identified in patients with intellectual disabilities with or without epilepsy (33).

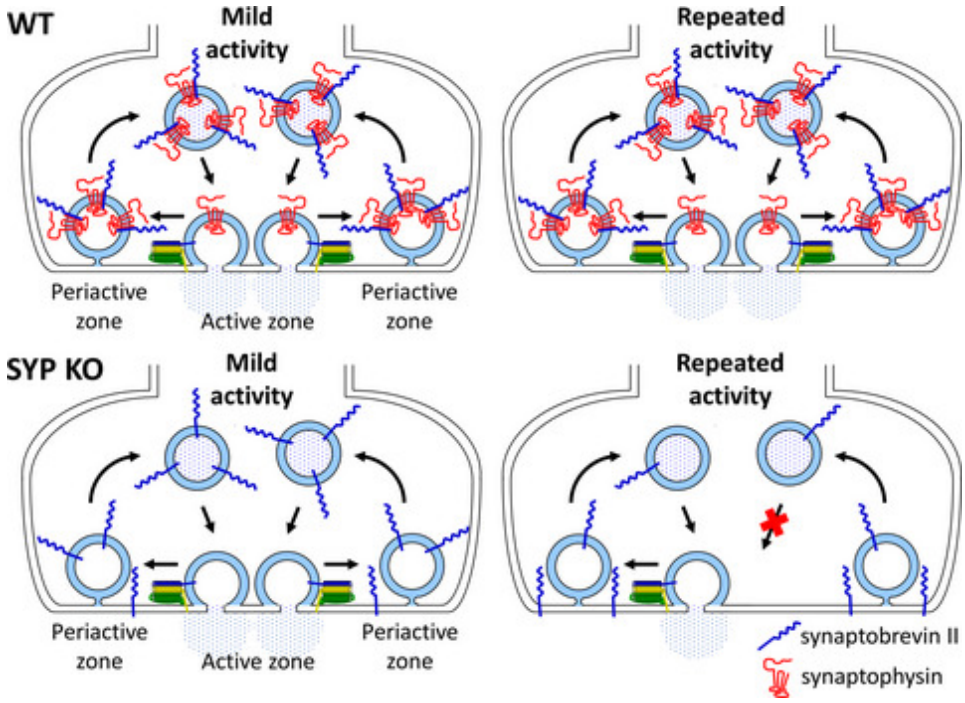

Figure 2 SYNAPTOPHYSIN ROLE IN SYNAPTIC NEUROTRANSMISSION
During periods of repeated synaptic vesicle turnover, the absence of synaptophysin in Syp knockout neurons results in an impaired synaptic vesicle trafficking and retrieval accompanied by a decrease in vesicular synaptobrevin II levels (28).

Furthermore, evidence has revealed synaptophysin involvement in plasticity-related changes during aging and after traumatic injury of hippocampal neurons, namely, synaptogenesis (34). One study has reported a significant age-dependent reduction in hippocampal Syp immunoreactivity specifically in the dentate gyrus and CA3 region, which are parts of a circuit critical for spatial learning (35). In contrast, increased Syp immunoreactivity has been detected in an Alzheimer mouse model, after chronic stress in hippocampal neurons, and after extensive behavioral characterization (36). These changes were speculated to be a result of a pathophysiological plasticity process implemented to compensate for cognitive decline in a stress situation (36-38). Moreover, Syp has been suggested to play an integral role in the generation of long-

term potentiation (LTP) in the hippocampal dentate gyrus (specifically, perforant path-granule cell synapses) as part of memory and learning processes (39).

Besides maintaining the flexibility and stability of individual neurons, homeostatic plasticity mechanisms can also act on a network level to stabilize circuit activity which is dependent on the functional equilibrium of excitation and inhibition (40).

B. Seizures and Epilepsy

Seizure, as defined by the International League Against Epilepsy (ILAE), is an irregular excessive or synchronous neuronal firing that leads to the occurrence of abnormal transient signs or symptoms (41). Seizures do not arise from abnormalities in a single neuronal population, but they rather result from complex interactions or malfunctions of different isoforms of receptors and voltage-gated channels within or between multiple brain regions. The exact mechanisms by which the neuronal system is pushed closer to seizure threshold are still under continuous investigation, however, most commonly, seizure-inducing malfunctions involve the inhibitory channels $GABA_A$ (gamma-amino-butyric acid) ionotropic receptors, NMDA receptors, AMPA receptors, and kainate receptors (KARs) (42). These triggers may result in increased overall excitability or inhibition or they may reflect physiological attempts to reestablish homeostasis which eventually causes a net shift in the electrophysiological homeostasis towards hyperexcitability. In clinical settings, a disruption in the brain's electrophysiological balance is usually displayed as abnormal electrical waves (spikes and sharp-waves components) on electroencephalography (EEG). A seizure on EEG is

characterized by sustained, abnormal evolving electrical activity that has a discrete electrographic beginning and end (onset and offset).

1. Seizure circuitry

A Suggested seizure basic wiring concept involves four major components of seizure evolution and spread: 1) a seizure focus; which is the region/regions of seizure onset and usually the major target for surgical interventions to prevent seizure occurrence. 2) Initiation circuits; which are neuronal networks that interconnect seizure focus with other vital parts to support and sustain seizure activity. 3) Secondary circuits or pathways responsible for seizure spread out of the initiation circuits by recruiting further networks leading to seizure generalization and widespread symptoms. And 4) Modulatory centers; are regions outside the initiation and secondary circuits that affect the likelihood of seizure occurrence and spread and regulate its severity and duration (43) (see figure 3).

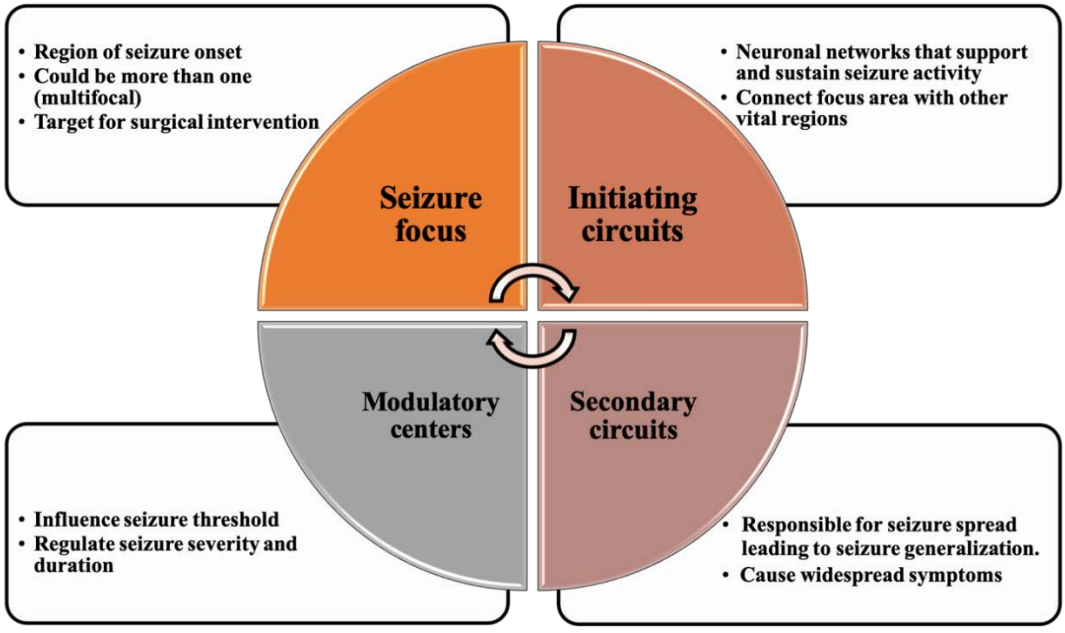

Figure 3 ILLUSTRATION OF FOCAL SEIZURES CIRCUITRY
The four suggested phases of focal seizure evolution and spread as a basic wiring diagram to understand the functional neuroanatomy of a seizure.

Based on the above circuit classification, seizures are categorized into generalized onset seizures, focal onset seizures, and seizures with unknown origin. Generalized seizures involve both hemispheres on onset whereas focal seizures originate in one hemisphere or one lobe and may spread to the contralateral hemisphere (secondary generalization) (44). Accordingly, clinical signs and symptoms accompanying each type of seizure depend on its respective region of origin. For example, a focal seizure arising from the occipital lobe in one hemisphere is expected to result in abnormal eye movements or elementary visual hallucinations on the contralateral visual field (45). On the other hand, focal temporal lobe seizures may manifest with loss of ability to speak, a sudden sense of fear or anxiety, epigastric sensations, repetitive behaviors and movements (automatisms), and déjà-vu illusions (46, 47). Notably, children are more prone to seizures than adults, especially infants. Their immature brain differs from that of adults in its susceptibility to seizures and epileptogenic mechanisms. They commonly display different clinical manifestations and unique EEG patterns of their seizures.

2. Epilepsy: recurrent seizure disorder

Epilepsy represents a number of debilitating chronic disorders characterized by recurrent unprovoked seizure activity. It is indeed one of the most common neurological disorders worldwide; affecting approximately 39 million people with a higher incidence occurring at the extremes of life (infancy and age >60) (48). Epilepsy can be genetic,

structural, metabolic, or idiopathic. Many brain assaults can contribute to an immediate or delayed seizure emergence and potential development of epilepsy. Traumatic brain injuries (TBIs), brain tumors, infections (such as malaria and bacterial meningitis), and strokes are risk factors for the development of acquired epilepsies (49). Moreover, some neurodevelopmental disorders and behavioral conditions might also be associated with an increased risk of epilepsy emergence such as autism spectrum disorder and attention deficit hyperactivity disorder (ADHD) (50, 51). Epilepsy is usually diagnosed after two or more unprovoked or reflex seizures occurring > 24 hours apart supported by electroencephalography (EEG), neuroimaging, and blood testing. Its management is aimed at controlling seizures with minimal adverse effects and supporting quality of life to reduce the probability of further complications such as physical injuries, cognitive deficits, long-term neurobehavioral disorders, social dysfunction, and reduced life expectancy.

Irrespective of the underlying etiology, epileptic conditions share a common end pathway of network hyperexcitability resulting from the disruption of the electrophysiological homeostasis (42). This imbalance between the main excitatory and inhibitory mechanisms is often detected as an EEG abnormality. Clinically, EEG is a monitoring method that measures ongoing neuronal voltage oscillations over a period of time (52). It reflects cerebral synchronous activity generated by a network of thousands or millions of neurons that are well-aligned and firing together. A normal EEG often displays low-amplitude, mixed-frequency, and symmetrical background waves (known as the posterior dominant rhythm) with transient physiological benign variants (52, 53) (see figure 4). On the other hand, an epileptic activity is shown on EEG as a synchronous evolving activity pattern with spikes, sharp waves, and/or spike-wave

discharges. Seizure type can be identified depending on the electrical pattern characteristics displayed on EEG and its accompanying clinical manifestations.

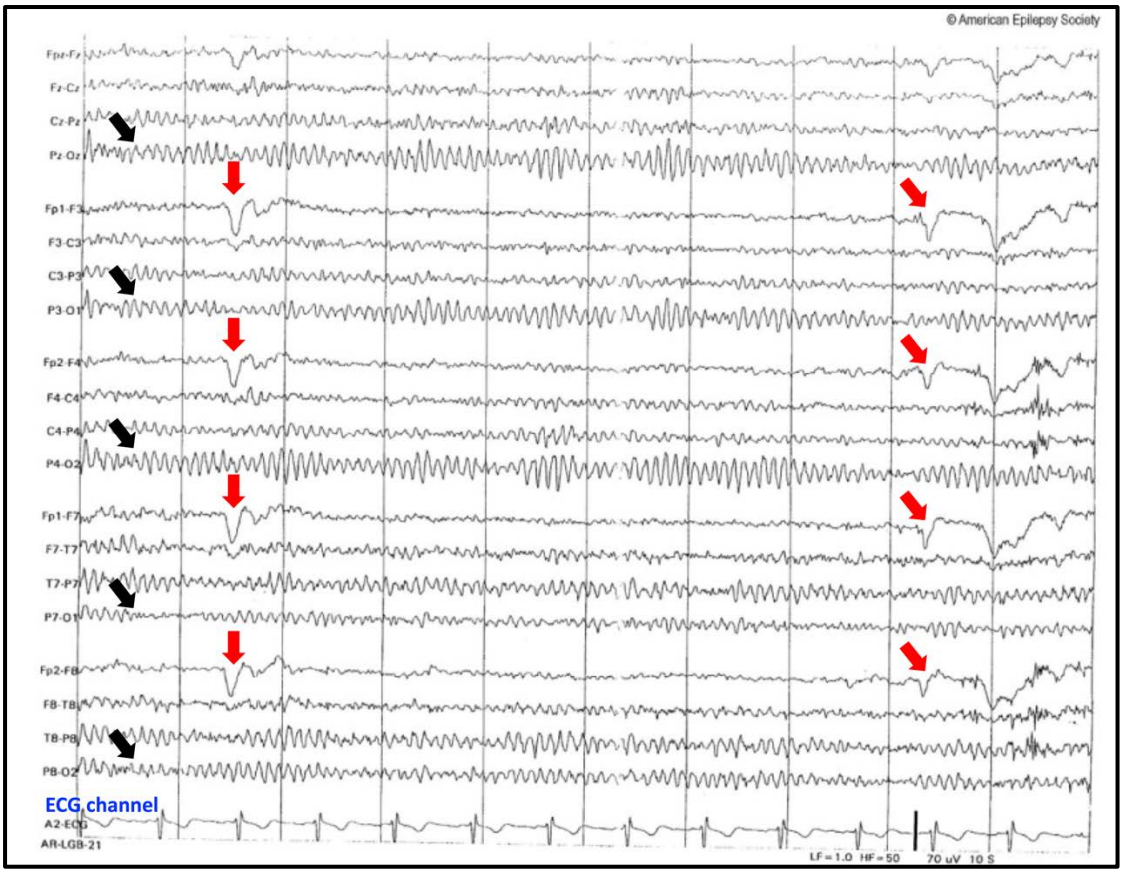

Figure 4 EXAMPLE OF A NORMAL HUMAN EEG RECORDED DURING WAKEFULNESS

Shown is a 10-second-long typical EEG montage of an awake 24 years old woman. Each division shows one second of recording time and every four channels (Fp-F, F-T, T-P, P-O) are referred to as a chain. Chains represent the cerebral activity of multiple head regions. The faster sinusoidal rhythmic activity in P-O channels (black arrows) is the posterior dominant rhythm (PDR). Benign physiological artifacts are indicated with red arrows. At the very bottom, an electrocardiograph (ECG) channel is displayed to help eliminating possible artifacts caused by cardiac dysrhythmias (53).

3. Temporal lobe epilepsy

Epilepsies can be divided into localization-related (focal) and generalized based on the mode of seizure onset (54). Most focal epilepsies are caused by structural brain abnormalities although these may not be always identified. Temporal lobe epilepsy

(TLE) is the most prevalent type of localization-related treatment-resistant epilepsies. TLE seizures arise mainly from mesial temporal lobe structures specifically; the hippocampus, amygdala, and parahippocampal gyrus (55). Clinical symptoms of focal seizures originating in the temporal lobe include behavioral arrest, mild ictal automatisms (repetitive, stereotyped, purposeless hand or mouth movements), staring, ictal speech and vocalizations, affective behaviors (laughing, crying, or fear), and postictal confusion which usually resolves within minutes (56). Patients with TLE often recall experiencing an "aura" preceding seizure characterized by an epigastric sensation (a "roller coaster" sensation), hallucinations (visual, olfactory, or gustatory), or psychic phenomena such as deja vu, jamais vu, or fear (57).

TLE is frequently associated with a wide range of psychiatric, cognitive, and behavioral problems that affect psychosocial functioning and quality of life. Although these problems can be attributed to medications used to control the condition, hippocampal sclerosis is the most common pathological finding in specimens of patients undergoing surgery for mesial TLE (58). Notably, hippocampal-related cognitive impairments such as deficits in learning, episodic and spatial memory, and emotional fear conditioning are the most common cognitive deficits associated with TLE (59-61). Fast high amplitude of > 50 μV transient EEG interictal spikes are frequently seen in patients with TLE. This interictal epileptic activity has been linked to abnormal GABAergic neuron recruitment arising in the hippocampus (62). In humans, TLE-related memory impairments have been directly linked to the occurrence of frequent interictal epileptiform EEG discharges and short non-convulsive seizures (63, 64). In addition, preclinical studies showed that the appearance of interictal spikes is associated with a decreased performance on recognition tasks and spatial memory tasks

in TLE rat models (65, 66). Moreover, TLE patients demonstrate multifocal neocortical atrophy, diffuse cortical hypometabolism, and numerous network perturbations that extend beyond the temporal lobe (67-70). Problems with concentration, social recognition, executive function, as well as depression and anxiety are common among TLE patients (71-74).

Evidently, most cases of TLE involve the dysfunction of the amygdala and hippocampus as major contributors to cognitive and emotional deficits. Their heavy interconnections contribute to their mutual involvement in TLE neuropathology.

C. Amygdalo-hippocampal function and circuitry

1. The hippocampus

The hippocampus, as a component of the limbic system, associates different kinds of highly processed information critical for daily life functioning. Humans and other mammals have two hippocampi within the anterior part of the medial temporal lobe on each side of the brain (see Figure 5). Each consists of cornu ammonis subfields CA1-CA3, the dentate gyrus, and the subiculum. However, most human studies include data from the more extended hippocampal region; involving the entorhinal, perirhinal, and parahippocampal cortices each of which is functionally distinct (75). In a cross-section of the hippocampus, several molecular layers (called strata) can be observed from the outer in; stratum lacunosum-moleculare, stratum radiatum, stratum lucidum, stratum pyramidale, and stratum oriens.

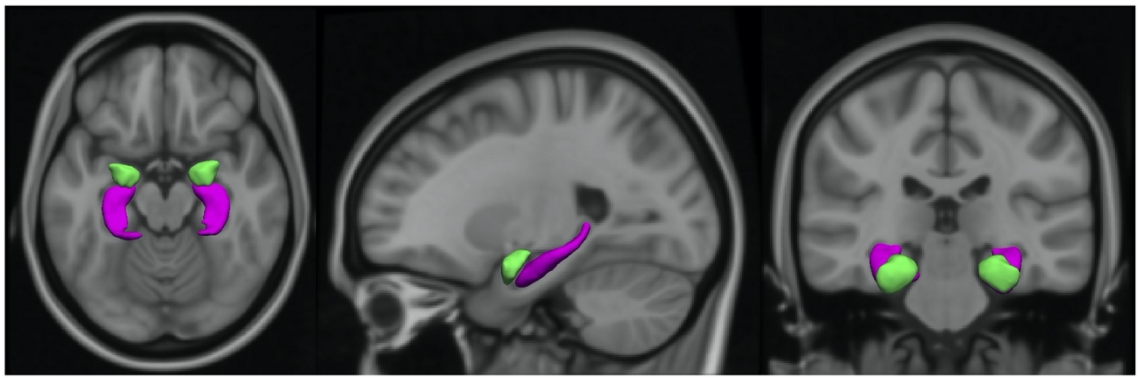

Figure 5 THE HIPPOCAMPUS AND AMYGDALA IN A T1-WEIGHTED MRI SCAN

From left to right, an axial, sagittal and coronal view of human brain showing locations of the hippocampus (purple) and amygdala (green) (76).

The hippocampus is a highly connected structure having widespread connectivity with several cortical and subcortical regions. Its input and output pathways have a laminar distribution in a major nonreciprocal unidirectional intrinsic network, namely the trisynaptic circuit initially described by Ramón y Cajal (77, 78). Much of the neocortical input the hippocampus receives runs through the entorhinal cortex reaching the dentate gyrus as part of a relay pathway called the perforant pathway. Granule cells; the main output cells of the dentate gyrus, give rise to axonal projections (called mossy fibers) connecting to pyramidal neurons of the CA3 subfield which in turn project to the CA1 subfield Shaffer collateral axons (5). Neurons of the CA1 subfield similarly send excitatory projections to the subiculum then into the deep layers of the entorhinal cortex for output (5) (see Figure 6). Accordingly, the perirhinal cortex is considered the gateway to the hippocampus since all extrinsic hippocampal interconnections with various cortical and subcortical structures such as the thalamus, hypothalamus, and brainstem, pass through the perirhinal cortex (5). Major types of neurons involved in these intrinsic and extrinsic hippocampal connections are excitatory

glutamatergic pyramidal neurons and inhibitory GABAergic nonpyramidal neurons (79).

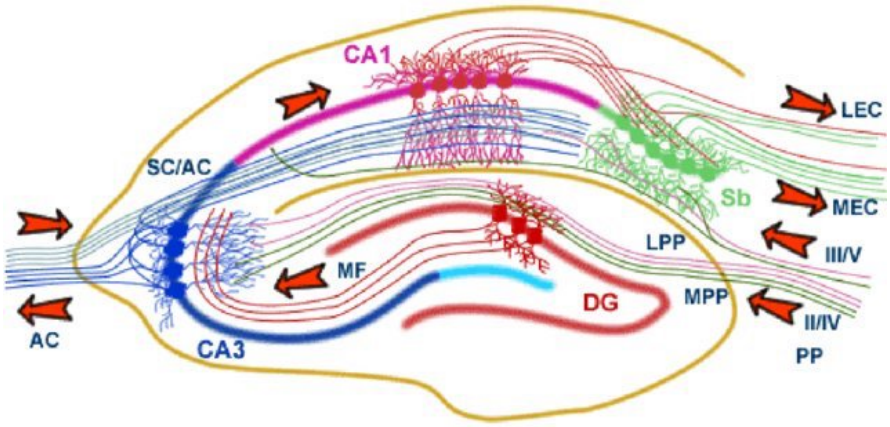

Figure 6 THE HIPPOCAMPAL INTRINSIC NETWORK
An illustration of the hippocampal trisynaptic circuit involving input and output connection pathways. Abbreviations: EC-Entorhinal cortex, DG-Dentate gyrus, SC-Schaffer collateral pathway, AC-Associational commissural pathway, PP-perforant pathway, Sb-Subiculum, MF-Mossy fibers, LPP-Lateral and MPP-Medial perforant pathway, LEC-Lateral and MEC-Medial entorhinal cortex, CA1 & CA3 subfields of the hippocampus. This figure is adapted from the University of Bristol website.

The hippocampus contributes to a wide array of behavioral and cognitive processes including, but not limited to, memory, spatial navigation, emotional processing, stress regulation, and decision making (80). It has functional specificity along its longitudinal axis and in between different developmental stages (75, 81). Most of the available neuroanatomical, functional, and molecular data on the hippocampus were obtained from studies conducted on rodents, mainly rats. As a polymodal association area, the hippocampus incorporates all sensory information into configurational neural representations of the surrounding environment (5). The ventral hippocampus in rodents, which corresponds to the anterior part in humans, is responsible for coarse, global representations. In contrast, the dorsal hippocampus, which corresponds to the posterior hippocampus in humans, is responsible for fine-grained, local representations (81, 82). Since the process of creating stable associations

must involve Hebbian plasticity between neurons (LTP and LTD), in this way, it has been speculated that the hippocampus generates interconnected maps of physical and mental space (83-85). Additionally, a growing body of evidence suggests a role for the hippocampus in pattern separation and approach-avoidance conflict processing in anxiety-provoking situations (86, 87). These functions require collaboration with other structures of the limbic system, specifically, the amygdala.

2. The amygdala

The amygdala is one of two almond-shaped groups of nuclei, each with distinct connectional and functional characteristics (88) (see figure 5). The amygdalae, located deep within the medial temporal lobes, are part of the limbic system and they share reciprocal connections with the hippocampus, entorhinal, perirhinal, and parahippocampal cortices.

Decades of research have shown that the amygdala is responsible for processing fearful and rewarding environmental stimuli and incorporating them into other aspects of cognition, particularly learning, memory, and decision making. Moreover, several electrophysiological studies have implicated the amygdala as a key modulator of hippocampal synaptic plasticity (LTP and LTD) necessary for the consolidation of emotionally-arousing memories (89). The amygdala receives convergent input from sensory association areas and thalamic nuclei, this information is integrated into aversive or reward stimuli giving them an emotional quality (5, 90).

3. Amygdalo-hippocampal dysregulation in diseases

Disruption of the amygdalo-hippocampal function is involved in many neurological diseases exhibiting cognitive and emotional deficits. For example, in Alzheimer's disease, impairments in declarative and emotional memory as well as fear conditioning result from the accumulation of neurofibrillary tangles and amyloid plaques in regions of the temporal lobe including the amygdala, hippocampus, and parahippocampal cortices (91). Another example is TLE; seizures originating in either the amygdala or hippocampus rapidly spread to the other. Multiple histopathological and imaging studies have reported concomitant neuronal damage in both structures in TLE (92). This damage contributes to impairments in emotional learning, fear conditioning, and associative memory (93). Evidently, repeated or prolonged seizure activity may induce anomalous neurogenesis in the dentate gyrus, specifically causing granule cell birth and survival (94, 95). Newly generated granule cells are known to be more excitable than their mature counterparts and more likely to show higher levels of synaptic activity markers in response to behavioral stimulation and experience (94, 96). However, with repeated seizure activity, newborn granule cells undergo premature differentiation, and integration into circuit networks promoted by seizure-evoked GABA-signaling. Consequently, their heightened plasticity is significantly decreased causing early retirement and diminished activation of these young granule cells contributing to further learning and memory impairments (97-100).

Recent lines of evidence support a role for the amygdala in many neuropsychiatric conditions such as depression, personality disorders, and aggressive behavior in epilepsy. Since the amygdala has a functional connection with brainstem respiratory centers, seizure invasion of the amygdala has been reported to induce partial

loss of spontaneous breathing (apnea) leading to the occurrence of sudden unexpected death in epilepsy (SUDEP); the most common cause of death in chronic refractory epilepsies (101). Furthermore, amygdalar hyperactivity is considered a major contributor to the symptoms of post-traumatic stress disorder (PTSD); a disease mainly caused by impairments in fear extinction and over-consolidation of fearful memories (102). The amygdalo-hippocampal circuit is involved in cued fear conditioning and extinction as a form of nondeclarative emotional memory. The dorsal hippocampus is responsible for sending contextual information to the amygdala for fear conditioning thus damage to the dorsal hippocampus has been reported to block fear conditioning of context-cued stimuli, but not to simple conditioned stimuli like tones (103). On the other hand, damage to the amygdala can cause dysfunctional fear conditioning to both types of stimuli (104). On this basis, decreased input from the hippocampus to the amygdala results in over-generalization of fear and the inability to differentiate between threatening and safe contexts (102, 105).

4. Behavioral tests used to assess amygdalo-hippocampal functions

A variety of animal behavioral testing panels have been used in neurobehavioral research to assess cognitive and emotional human traits such memory, social interactions, anxiety, depression, and learning (106). These tests are designed to mirror human conditions and are conducted in controlled environments to give them the validity to be translated into clinical settings. To name a few, the light dark box (LD) and open field (OF) tests are used to assess anxiety-like and exploratory behaviors, the forced swim test (FST) is employed to examine struggling and depressive-like behaviors, and the Morris water maze (MWM) which is a test for hippocampal-

dependent visuospatial navigation (see table 1). These tests are commonly utilized in investigations of long-term consequences of induced CSE on emotional and behavioral functions with adjustment to be more objective and measurable. In TLE models of SE, decreased struggling and depressive-like behaviors were observed in the FST, anxiety-like behaviors and hyperactivity in the OFT, and abnormalities in visuospatial learning in the MWM (107).

Testing panels	Used for	Measurable behavioral responses
Forced swim test (FST)	Depressive-live behaviors	Time spent immobile swimming and struggling activities, climbing attempts, latency time to the onset of immobility
Open field test (OFT)	Exploratory, hyperactivity, and anxiety-like behavior	Distance traveled, time spent in each zone, time spent exploring central objects, time of immobility (freezing), speed in each zone
Light-dark-box test (LDT)	Anxiety-like behavior	Time spent in each compartment, latency time to first transition, number of transitions between the two compartments, exploratory activity levels reflecting time spent next to novel objects on the lit side
Morris water maze (MWM)	Visuospatial navigation	Escape-directed swimming behavior (escape latency, distance traveled)
Modified activity avoidance (MAAV)	Emotionally-relevant learning for auditory and contextual cues and adaptive shock-avoiding behaviors	Percentage of avoidance and latencies (time required to avoid the shock before Its onset upon perceiving the conditional stimulus)

TABLE 1 BEHAVIORAL TESTS COMMONLY USED IN OUR LABORATORY
Tests used for assessments of amygdalo-hippocampal functions in epilepsy rodent models with prominent limbic involvement.

The MAAV test is designed to simultaneously assess the recognition of auditory and contextual emotional cues and the acquisition of learned adaptive shock-avoiding

behaviors. This test is one of the main panels used in this study to assess the functionality of the amygdalo-hippocampal circuitry following intrahippocampal chemical (kainic acid) administration. It is a modified version of the classical conditioning test, derived from Pavlovian conditioning that involves a conditioned stimulus (CS) that can be represented by either contextual visual cues, lights, or more commonly a tone, and an aversive unconditioned stimulus (US) such as an electrical foot-shock (108). This test allows for testing of learned adaptable responses characterized by anticipation and hence, avoidance of an unconditioned aversive stimulus. Fear of painful foot-shock is a survival-related innate reaction in rodents that elicits a response of freezing upon the initial recognition of CS as being a threat (109). This innate response is replaced by an acquired avoidance response in instrumental conditioning as in the MAAV test.

D. Prolonged convulsive and non-convulsive seizures

1. Status epilepticus

When a seizure activity continues for a sufficiently prolonged period, the patient is said to have status epilepticus (SE), one of the most common life-threatening neurological conditions. It is thought to be caused by the failure of endogenous mechanisms responsible for seizure termination resulting in an irreversible neuronal damage worsened with increased duration. During a seizure, synaptic trafficking increases along axonal membranes in the epileptic focus resulting in a gradual depletion in functional inhibitory $GABA_A$ receptors, an increase in the number of excitatory glutamatergic NMDARs transported to synaptic membranes, and alternations in excitatory and inhibitory neuropeptides expression (110, 111). These maladaptive

changes maintain the hyperexcitable state and allow a single seizure to become persistent and self-sustained. However, much of the basic mechanisms underlying SE is still poorly understood. Patients who experience only one episode of a prolonged seizure are estimated to have a 10-30% chance of a lifetime recurrence and 20-40% risk of developing epilepsy (112, 113). These prolonged seizures may involve variable levels of motor involvement and altered consciousness. Accordingly, SE has been classified based on its electrophysiological mode of onset and clinical presentation into convulsive SE (CSE) and non-convulsive SE (NCSE).

The approximate timepoint after which a seizure will most probably persist or recur and inflict damage differs according to the type of seizure (41). It is well-established that if a motor convulsive seizure lasts beyond 5 minutes, anti-seizure treatment must be provided and sometimes sedation might be needed to abort it and prevent imminent brain damage, which is known to occur after 30 minutes of continued or intermittent seizure activity without recovery (returning to neurofunctional baseline). On the other hand, data regarding seemingly benign non-convulsive seizures are limited and the duration required to cause brain damage (if any) is still unknown.

Convulsive SE is associated with significant morbidity and mortality. In contrast, reports of incidence and outcomes of NCSE are contradicted, due to frequent misdiagnosis and the existence of several contributing acute medical etiologies. Meanwhile, some postulated that it accounts for up to 20% of all cases of prolonged seizures in general hospitals and up to 47% in the intensive care unit (114, 115).

Despite being first described in the mid-1800s, NCSE is still an underreported, underdiagnosed and understudied condition. Moreover, most of the used treatments are extrapolated from studies of the convulsive type which has well-established guidelines

for treatment. Additionally, clinical studies focusing on NCSE are often being conducted on critically ill patients continuously monitored by EEG, leaving a huge gap in knowledge of its behavioral and neuropathological consequences in ambulatory patients who are typically susceptible to delayed diagnosis or inadequate treatment whenever they realize their need for medical attention.

2. Non-convulsive status epilepticus (NCSE)

Non-convulsive seizures refer to all types of signs and symptoms that result from a transient abnormal neuronal activity that is not accompanied by tonic muscle stiffening or clonic rhythmic activity. Accordingly, the term non-convulsive status epilepticus (NCSE) refers to prolonged electrographic seizure activity that involves minimal or no motor symptoms and variable levels of altered consciousness. This term has been used to describe different conditions with diverse underlying pathophysiologies, levels of consciousness, and EEG patterns. NCSE accompanied by severely impaired mental state or coma (previously known as subtle SE) usually develops after inadequately treated generalized convulsive SE. In a report published by the ILAE task force, NCSE, when it is not accompanied by coma, was further classified into 3 subtypes: generalized absence NCSE (typical, atypical and myoclonic SE), localized focal NCSE (also known as complex partial SE), and a subtype that is unknown whether it is of generalized or focal origin (e.g. autonomic SE) (41) (see Figure 7).

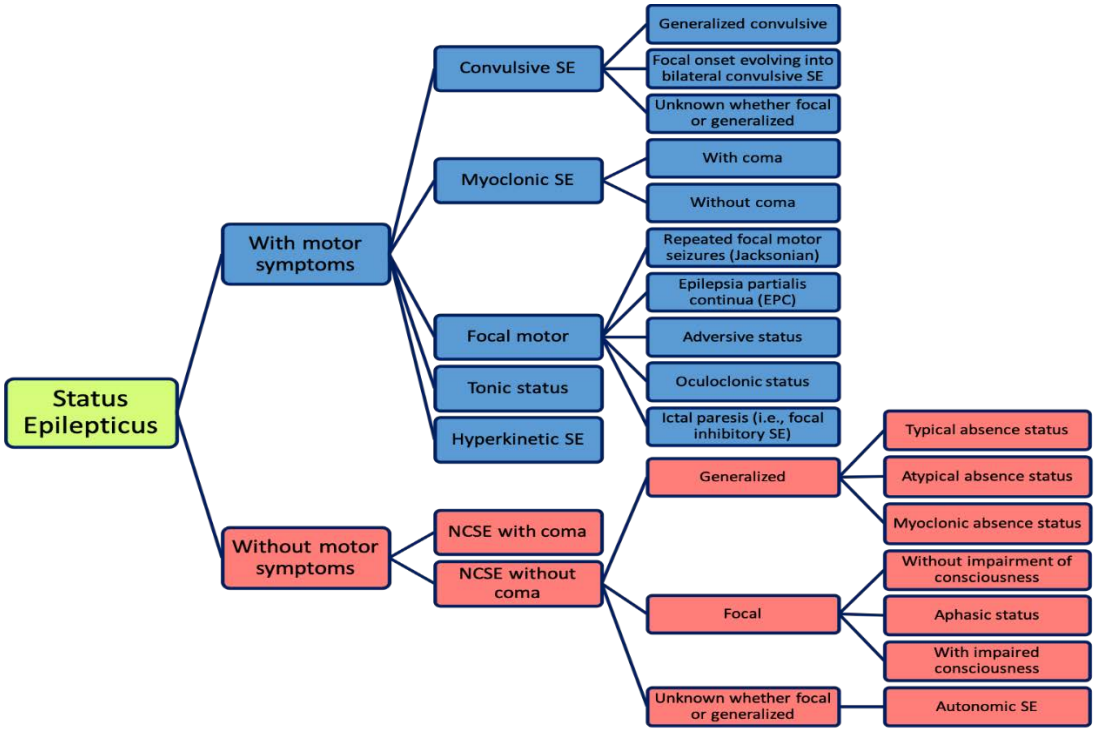

Figure 7 STATUS EPILEPTICUS CLASSIFICATION
SE classified based on its electrophysiological mode of onset and clinical presentation. Modified from the 2015 ILAE published report on definition and classification of status epilepticus (41).

Depending on its pathophysiogenesis, NCSE can be symptomatic or cryptogenic and its outcomes are influenced largely by underlying etiologies which are varied and differ according to the patient's population being studied. NCSE might be acquired due to infections (e.g. infectious or autoimmune encephalitis), structural-metabolic disorders (e.g. ischemic or hemorrhagic strokes, neoplasms, traumatic brain injuries, hippocampal sclerosis, and vascular or cortical malformations), intoxications (such as Domoic acid, star fruit, cocaine, ecstasy, and lead intoxications), inflammatory disorders, or sleep deprivation (116). Also, it might be neurogenetic in nature and caused by mitochondrial diseases or other genetic causes (41, 117). Whereas more than 30-50% of cases presenting with NCSE have a prior history of some form of seizures or epilepsy (118).

3. Pathological and behavioral consequences of NCSE

It is hard to differentiate between molecular mechanisms contributing to the occurrence of prolonged seizures and those resulting from it. Reliable reports of any cellular or structural changes due to NCSE in humans are still lacking. On the other hand, it is well-established that convulsive SE causes acute neuronal damage predominantly in the hippocampi, thalami, and neocortex (119). Even if motor convulsions are avoided by induced muscle paralysis, electrographic seizures still result in neuronal cell loss suggesting that NCSE also has the potential to cause deleterious effects on the brain (120). The excitotoxic effects of glutamate, calcium and possibly zinc influx into neurons, oxidative stress and mitochondrial dysfunction may seem to be the major contributors to any induced neuronal damage (121). The damage can be wide-spread and may involve areas distant from seizure focus (122). One study reported a four-fold increase after NCSE in serum neuron-specific enolase, a marker of acute neuronal injury, in patients with no acute neurological insult (123). This increase is thought to be generated by a dysfunction in blood-brain barrier caused by seizure-induced cytotoxic edema, transient morphological changes of astrocytes, reactive gliosis, and angiogenic processes (124, 125). Moreover, leakage of serum albumin and IgG into the brain and associated neuroinflammatory response in permeable regions, specifically the piriform cortex, are considered predictors of epileptogenesis (125). Additionally, the induced changes in perineuronal micro-environment, neuronal network rewiring, and pathological synaptic plasticity all contribute to increased excitability and lowered seizure threshold (126-128). In-vivo studies have provided evidence that prolonged seizures lead to brain injury, however, in chemically induced seizure models, it is hard to differentiate between molecular changes resulting from the

exogenous proconvulsant mechanisms being used and those provoked by the prolonged seizure activity.

Few but alarming reports point to several cognitive and behavioral disturbances attributed to non-convulsive seizures. A cross-sectional study was done on 188 children to assess the effects of short non-convulsive seizures on learning and cognition, it revealed clinically significant impairments in short-term memory, language, visuospatial functions, and speed of central information processing (63). Additionally, interictal epileptic spike patterns are seen frequently in animal models of chronic post-SE and epileptogenesis models and usually precede seizure activity. These interictal electrical activities were associated with deteriorations in IQ performance, expressive and receptive language, and numerical skills in children with focal epilepsies (129-132). This observation was strengthened by significant long-term improvements in IQ scores, psychosocial functions, and quality of life in children who had temporal lobectomy to treat their TLE, suggesting that cognitive deficits previously observed were transient and associated with the ictal activity itself and the anti-seizure medications used to control it rather than its underlying etiology (133). Another study reported acute impairments in alertness, attention and short-term memory function following recurrent short non-convulsive seizures (134).

E. Attempts to study NCSE in animal models

Most of the kindled animal models of seizures, or epilepsies in general, are highly predictive and not clinically validated. There is not one “ideal” animal model for the study of NCSE, however, it is known that NCSE mostly originates in the temporal lobe on one hemisphere and spreads into the other, thus its characteristics may be

efficiently reproduced in animal models of temporal lobe epilepsy (TLE), which is the most common type of epilepsy. TLE typically results from structural and/or functional epileptogenic alterations in the limbic system, particularly involving the hippocampus, amygdala, and entorhinal cortex (135, 136). These structures are thought to have the lowest threshold, among other limbic components, for seizure development and maintenance (137). Usually, evoked non-convulsive seizures involving these areas manifest in animal behavior in a way that mimics several characteristics of human seizure expression (138). Nevertheless, detecting these behavioral abnormalities, such as automatisms, in experimental animals is difficult and can be easily over-interpreted therefore, it should be always correlated with EEG recordings. One of the pitfalls of using animal models in epilepsy research, in general, is that predicting prognosis of a condition, like NCSE, can be faulty due to the hyperexcitatory nature of induced seizures which might not exist in humans (139). Another one is that cognitive states in rodents, which are the most commonly used animals in epilepsy research, are much less complex than those of human beings decreasing their inferential value.

Since it was first described in 1973, the Racine scoring system has been used to evaluate seizure intensity based on animal behaviors (140). However, this system was developed originally for the electrical amygdala kindling model thus it required multiple modifications to better describe other experimental seizure models. Accordingly, limbic SE is further classified in 4 types: type I (referred to as immobile status) in which the animal shows motionless persistent staring and the status is only detectable by EEG recording. Type II (referred to as exploratory or ambulatory status) where the animal shows abnormal discontinuous exploratory behaviors corresponding to stages I and II on Racine's scale. Both types I and II are sub-convulsive and involve

the amygdalo-hippocampal area and some of its efferent projections. Type III (reffered to as masticatory status) in which the animal starts to show mild to severe facial and forelimb clonus and automatisms. Type IV referred to as clonic status or generalized status) in which continuous generalized clonus is observed (137) (see Table 1). Based on this classification NCSE might range between types I, II and III according to its behavioral manifestations and EEG correlates (140).

Limbic SE classification	Common name	Score on Racine scale [140]	Behavioral manifestations
Type I	Immobile status	1	Motionless persistent staring
Type II	Exploratory or ambulatory status	1,2	Abnormal discontinuous exploratory behaviors
Type III	Masticatory status	3	Mild to severe facial and forelimb clonus and automatisms (wet dog shakes (WDS), sniffing, repeated washing, scratching, or excessive orientation behavior with repeated rearing)
Type IV	Clonic status or generalized status	4,5	Continuous generalized clonus

Table 2. STATUS EPILEPTICUS CLASSIFICATION BASED ON THE RACINE SCALE

Table showing limbic SE classification used to determine seizure severity in experimental animal models.

Although not relevant etiologically, several chemical and pharmacological agents are being used in epilepsy research based on their cellular mechanisms and pharmacodynamics. Usually, those used either work by enhancing excitatory neurotransmission, or by blocking inhibitory mechanisms to evoke seizures. These agents can be administered systemically or focally. However, Intracerebral administration is preferred over systemic administration in order to avoid undesirable peripheral side effects of the chemoconvulsant agent which may affect the model's resemblance to the human disease state. Most often, male rodents are preferred over females in such models because female's sensitivity to chemoconvulsants is affected by

hormonal factors (e.g. estradiol) (141). Moreover, focally applied chemoconvulsants inflict consistent patterns of damage restricted to specific brain areas which improve the reproducibility of the model and decrease mortality rates significantly. The duration of the induced SE is usually controlled pharmacologically in order to obtain a reliable chronic post- SE model.

1. Kainic Acid (KA) induced seizure model

Kainic acid, a synthetic cyclic analog of L-glutamate, is a powerful excitotoxic agent usually injected systemically or into the dorsal hippocampus or amygdala to evoke a prolonged seizure. It binds to the kainate receptors, an ionotropic glutamate receptor type highly expressed in the hippocampus, amygdala, perirhinal and entorhinal cortices (142). These receptors are involved in the modulation of inhibitory and excitatory neurotransmitter release and synaptic network activity regulation (143). Activation of kainate receptors is known to cause seizures arising from the limbic structures (144). These seizures are characterized by behavioral arrest or immobility with automatisms, such as wet-dog shakes, repeated washing, sniffing, or scratching, progressing in a dose-dependent manner into convulsive behaviors but they rarely reach a tonic-clonic stage (138). Doses applied can range from 7 ηg up to 0.4 μg for intra-hippocampal injections and 0.1 μg up to 2 μg for intra-amygdaloid injections (145-148).

KA models have been used since the early 1980s, but they became more popular in the late 1990s when it was reported that mice receiving intrahippocampal kainite injections develop spontaneous recurrent seizures and hippocampal sclerosis later in life (149). Recently, they found that unilateral intrahippocampal injection of a low dose KA induces an initial neurotoxic NCSE event that may last for hours followed by a latent

phase lasting between two to three weeks. After this phase, spontaneous recurrent hippocampal paroxysmal discharges (HPD) start to appear on EEG arising from the lesioned hippocampus and become persistent after 4 weeks of the injury and for the life of the animal (149-151). EEG recording after KA intrahippocampal injection shows spikes, polyspikes, and spike-and-wave discharges at high amplitude (similar to patterns seen in humans during an NCSE) lasting for several hours (152). Intracerebral administration of KA is known to result in a severe ipsilateral neuronal damage, reactive astrogliosis, granule cell dispersion, mossy fiber sprouting, synaptic reorganization, and hippocampal sclerosis comparable to those observed in TLE patients (149, 153). These direct neurotoxic effects are mostly observed (when KA is delivered into the hippocampus) on pyramidal neurons of CA1 and CA3 regions of the hippocampus, the dentate hilus, and even outside (154). Another observation was increased expression of kainate receptor subunits in reactive astrocytes of the hippocampus and surrounding cortex during the latent phase after SE induction, which may pose as a potential contributor to the epileptogenic process and the emergence of spontaneous behavioral seizures (153).

KA is also frequently administered systemically in doses of 10-60 mg/kg i.p. to induce SE in rodents. Seizures induced by systemic KA are reported to originate in the entorhinal cortex and spread via the perforant pathway into the hippocampus (144). However, this method results in higher mortality rates compared with intracerebral administration. Such an issue is often dealt with by dividing the required dose into smaller increments given gradually over an hour or two to account for variation in sensitivity between the same species and to assure maximal survival (155). It was argued that rodents receiving intrahippocampal KA are much less aggressive and easier

to handle than those receiving systemic injections of KA or pilocarpine (154). Even so, this method has several limitations such as the disruption of the blood-brain barrier caused by canula placement surgery and microinjection procedures, the intrahippocampal KA model has great advantages for long-term studies of epileptogenesis post-SE and investigating potential pharmacotherapeutic and neuroprotective agents.

2. A novel model of temporal lobe NCSE established at our laboratory

In our laboratory, a model of induced-NCSE was recently established using a subconvulsive intrahippocampal dose of 0.00625 μg of KA in peri-adolescent rats that recapitulates the electroclinical features of temporal lobe NCSE. In this model, KA-induced NCSE behavioral manifestations included oromotor automatisms such as chewing and licking, in addition to behavioral arrest, staring and unresponsiveness, without progressing to convulsive signs (Racine scale 4 or 5) resembling those of human condition. In addition to its face validity, this model is highly reproducible with a high success rate of NCSE induction reaching 90% and no mortality.

Furthermore, in a pilot study conducted at our laboratory using this model to investigate potential NCSE-induced effects on emotionally-relent learning and memory indicated that multiple episodes of prolonged NCSE of a temporal lobe origin have a negative influence on learning and memory in rodents, specifically, hippocampal-dependent learning and contextual-cued shock avoiding behaviors. In addition to depressive-like behaviors noticed following controlled repetitive episodes of induced NCSE confirmed by EEG recording.

Here we utilize this model for the aims listed in the following chapter.

CHAPTER II

AIMS AND HYPOTHESES

NCSE remains an under-investigated epileptic condition. In clinical settings, NCSE is being misdiagnosed, undertreated, or in some cases overtreated given that it lacks overt convulsive activity and requires a high index of suspicion. However, accumulating reports have been pointing to potential NCSE-related harmful effects on the central nervous system. Knowledge of NCSE's mechanisms and consequences may encourage the implementation of consistent guidelines for its diagnosis and management. Considering that NCSE commonly originates from the temporal lobe in adolescents and adults, we use our recently established peri-adolescent rodent model of temporal lobe NCSE to investigate its acute effects on amygdalo-hippocampal structural and functional integrity. In addition, we examine potential late NCSE-induced cognitive and behavioral changes, as delineated below.

Aim 1: To confirm our preliminary outcomes indicating the emergence of potential early deficits in cognitive and emotional learning in the modified active-avoidance test (MAAV) following KA-induced temporal lobe NCSE.

Hypothesis 1: KA-induced temporal lobe NCSE will negatively affect the amygdalo-hippocampal circuit functionality leading to impairments in emotionally-relevant cognitive and learning behaviors.

Aim 2: To investigate the underlying mechanisms of potential NCSE-induced learning deficits by assessing neuronal density, glial fibrillary acidic protein (GFAP) expression, and changes in the synaptic plasticity marker, synaptophysin (Syp) protein levels.

Hypothesis 2: One or two episodes of temporal lobe NCSE will potentially evoke neuronal hippocampal damage (neuronal cell loss and reactive astrocytosis) and maladaptive alterations in synaptic plasticity. In the same way, the enhancement in learning previously observed after one episode of NCSE might be caused by a beneficial spur in synaptic plasticity.

Aim 3: To check for the persistence of previously observed memory and learning deficits in the MAAV test one month post-KA-induced NCSE and to investigate NCSE-related potential later life effects on visuospatial memory (Morris wate maze), anxiety-like behaviors (Light-dark box and open field tests), and depressive-like behaviors (Forsed-swim test).

Hypothesis 3: Temporal lobe NCSE-related hyperexcitability will disrupt the normal amygdalo-hippocampal circuitry potentially resulting in long-term alterations in functions known to be dependent on these structures' integrity including learning and memory, spatial navigation, stress regulation, and emotional processing.

CHAPTER III

MATERIALS AND METHODS

A. Animals and experimental design

Male Sprague-Dawley rats obtained from the animal care facility at the American University of Beirut were housed under constant conditions of temperature (20-23 °C) and lighting (A normal 12:12 hour light-dark cycle). Animals had access to regular rat chow and water ad-libitum except during behavioral testing or when scheduled for sacrifice. Epidural electrodes and cannula implantation surgery was performed at postnatal day (P35) as described below (see section III-B), and all rats were allowed 4-7 days of recovery before seizure induction with proper pain management. All procedures were approved by The Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut and all efforts were made to minimize discomfort throughout the duration of experimentation. Two paradigms were applied, a short-term one and a long-term one, with a total group number of n=18-23 rats in the short-term paradigm and n= 5 in the long-term one.

In the short-term paradigm: At postnatal days (P43-P44), animals were subjected to one or two episodes of NCSE (via intra-hippocampal kainic acid (KA) injections) or sham manipulated under continuous EEG monitoring as described below (see sections III-C and III-D). 24 hours following the second injection, rats were subjected to the modified active-avoidance test (MAAV) for 7 consecutive days and were sacrificed on the last day of testing, at P52, during the 30-90 minutes post-testing Period. Paraformaldehyde (PFA) perfused brains were collected for histological analyses (see figure 8).

In the long-term paradigm: After NCSE seizure induction (at P43-P44), rats underwent continuous EEG recording for one month. Behavioral testing was started at P72 for 30 consecutive days then rats were sacrificed 30-90 minutes post-MAAV testing at P91. PFA perfused brains were collected for histological analyses (see figure 8).

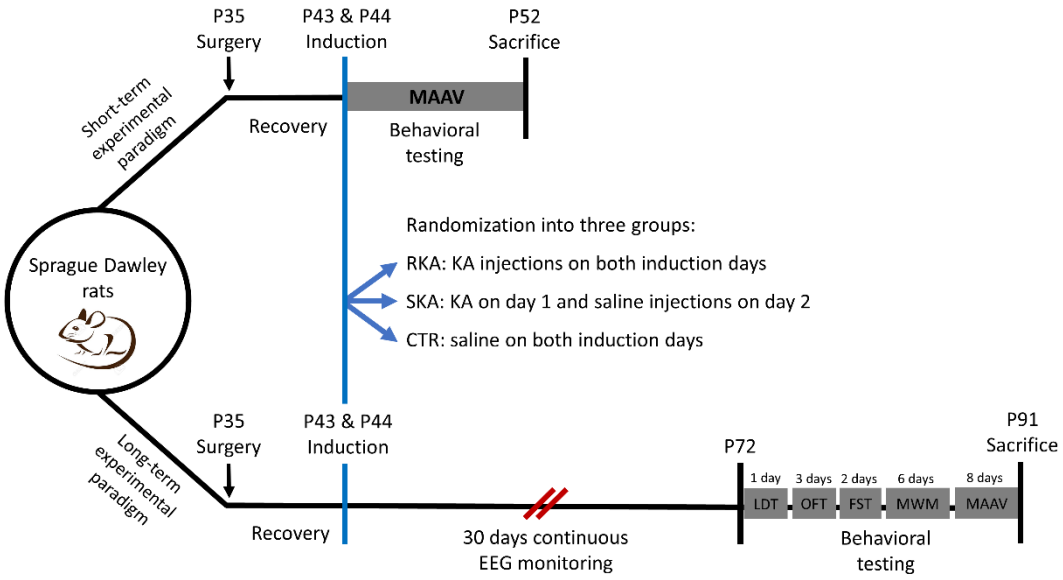

Figure 8 SCHEMATIC STUDY DESIGN
Sprague-Dawley peri-adolescent male rats were used for both paradigms. Epidural electrode and cannula implantation surgery was performed at postnatal day (P35). After being 4-7 days of post-operational recovery, at P43-P44, NCSE seizure induction was performed under continuous EEG recording as follows: RKA group received two 24-hour apart KA injections, SKA group received a KA injection at P43 and a normal saline injection at P44, and CTR group received two 24-hour apart normal saline injections. For the short-term paradigm: behavioral testing was initiated at P45 with the modified active-avoidance test and rats were sacrificed 30-90 minutes following MAAV retention testing at P52. For the long-term paradigm: Behavioral testing was initiated after approximately one month of prolonged continuous EEG monitoring at P72 and rats were sacrificed 30 minutes following MAAV retention testing at P91.

B. EEG electrodes/cannula implantation surgery

Epidural electrode and cannula implantation surgery was performed at P35. Proper anesthesia was achieved using an intraperitoneal injection of a mixture of

Ketamine (80 mg//kg) and Xylazine (10 mg/kg) and maintained when needed throughout surgery with half or quarter of a dose through intramuscular administration. After shaving the surgical site from the flat of the nose between the eyes down to the neck, the rat was placed on a pad with the head tightly secured on a stereotaxic frame. To prevent dryness or possible irritation of the eyes, a lubricating eye ointment was gently applied. After sterilization of the scalp with iodine fallowed by ethanol, a straight midline incision was made using a surgical blade, and the skull was exposed using a retractor. The periosteum connective tissue that adheres to the bone was scraped and cauterized to minimize bleeding and insure sufficient dental acrylic cap adherence to the skull. A high-speed stereotaxic drill was used to make five small 1.4 mm holes in the skull for epidural electrode placement. These included left and right frontal electrodes (F3 and F4: 2 mm anterior to, and 3 mm lateral to the bregma), left and right parietal (P3 and P4: 5 mm posterior to, and 3 mm lateral to the bregma) and one anterior midline reference electrode (Ref: 6 mm anterior to the bregma) based on the Sherwood and Timiras Stereotaxic Atlas of the Developing Rat Brain (156) (see Figure 9).

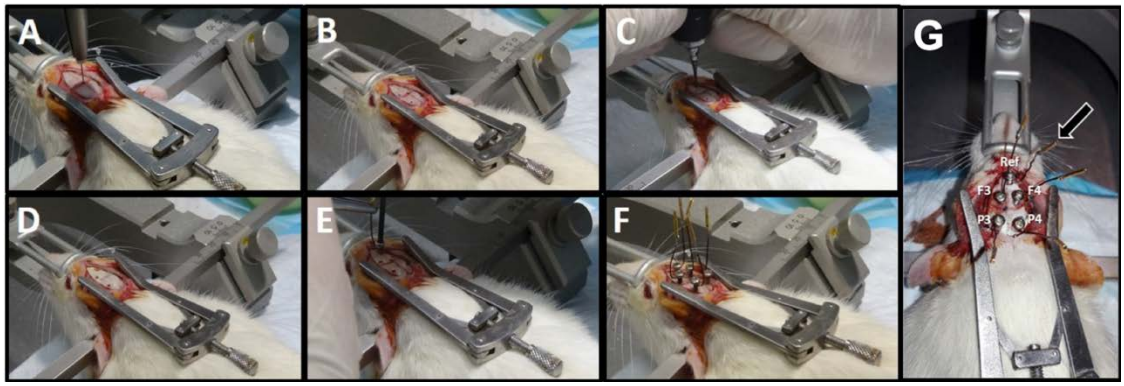

Figure 9 ELECTRODE/CANNULA IMPLANTATION SURGERY
Panel A: After exposing the skull, the bregma is identified with the stereotaxic arm and its antero-posterior and lateral coordinates are recorded. **Panel B**: the desired locations of the five reference and sampling electrodes and the cannula are calculated and marked based on the Bregma's coordinates. **Panels C and D**: a high speed drill held perfectly vertical to the skull's surface is used to make six small holes for the electrode screw attachment and cannula insertion. **Panel E**: electrode screws are equally

inserted using a screwdriver. **Panels F and G**: Shown are the placed electrodes (two frontal; F3 and F4, two posterior parietal; P3 and P4, and a reference electrode; Ref).

An extra hole was made for the intrahippocampal guide cannula (2.6 mm in length) which was implanted stereotaxically onto the CA1 region of left hippocampus (2.4-2.7 mm posterior, and 2 mm lateral to the bregma) (see figure 10).

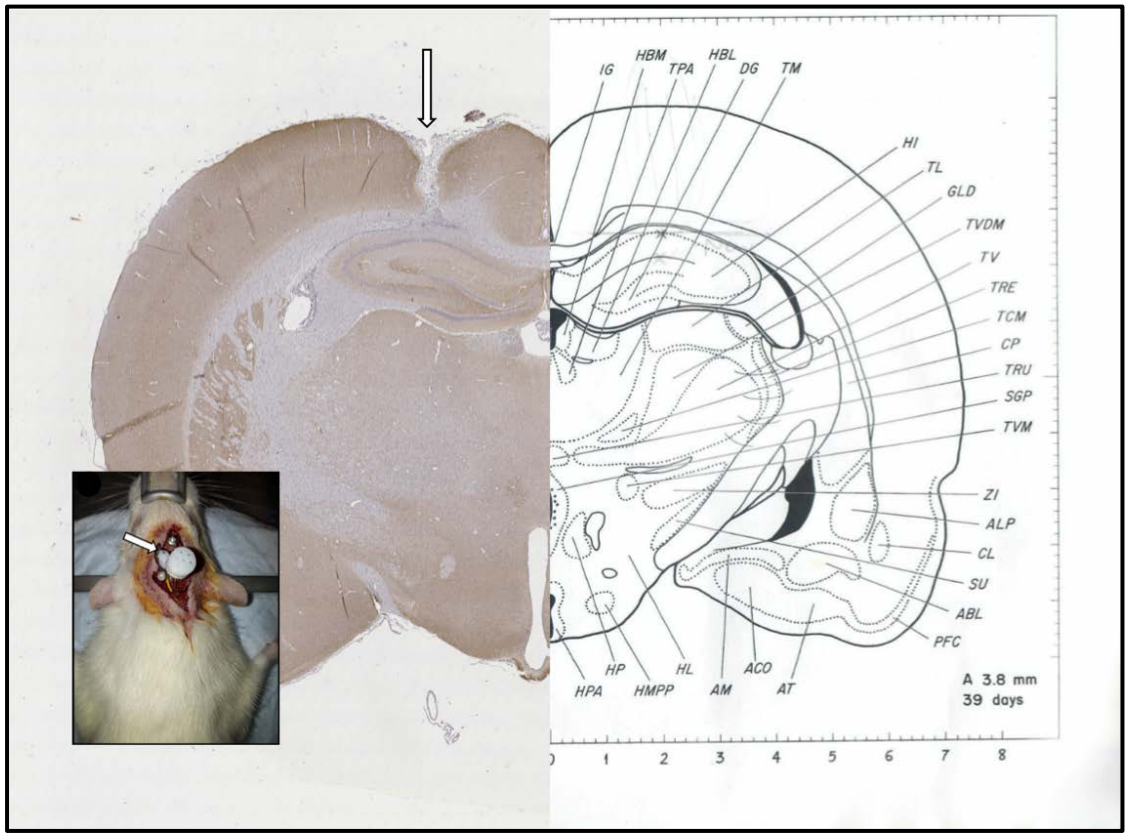

Figure 10 INTRA-HIPPOCAMPAL CANNULA LOCATION ON THE ATLAS OF THE DEVELOPING RAT BRAIN
Shown is the cannula placement location above the left hippocampus on Sherwood and Timiras Stereotaxic atlas and an image of assembled electrode-pedestal set to the right of the cannula (white arrow) (156). (bregma is at A 6.2 ± 0.5 mm)

The pins extending from the electrode wires were then inserted in a 6-channel size appropriate pedestal (Plastics One, USA) along with a 6th socket attached to a free wire placed under the skin at the base of the neck to serve as the ground electrode. The headset was then covered by dental acrylic cement to give it a final cap-like shape. Rats

were then transferred to customized single-animal cages for observation. Analgesic treatment with Paracetamol was administered via drinking water (1 mg/ml) for three days postoperatively. All rats were allowed 4-7 days to recover before seizure induction.

C. Continuous EEG recording

Baseline brain activity EEG recording was initiated on seizure induction day at least 2 hours prior to kainic acid administration. Rats were monitored in customized nonconductive Plexiglas EEG cages that provide the necessary electrical isolation from sources of ambient electricity. Implanted electrodes were attached to the EEG recording system (Xltek, Natus Medical, USA) using an enhanced customized swivel-balance EEG-cage system that accommodates the movement of the rats. (157) (figure 11)

Figure 11 LIVE VIDEO-EEG RECORDING SETUP
Rat's electrode/cannula headset is attached to an EEG cable equipped with a commutator that allows full rotation to accommodate rat's movement (**Panel A and B**). Rats are placed in customized vertical electrically isolated Plexiglass cages (**Panel C**). The full setup of the EEG system is shown in **panel D**.

In both paradigms, EEG recordings were reviewed and quantified by two readers blinded to treatment groups. NCSE durations and latencies were determined through times of electrical and behavioral seizure onsets and offsets following kainic acid injections. Moreover, long-term EEG recordings were reviewed to check for

possible seizure recurrence or abnormal electrical patterns, e.g. spikes (a pointed peak followed by a repolarization wave) and poly-spikes (two or more spike components). Spikes and polyspikes were quantified in randomly sampled 8 hours per day (4 from daytime and 4 from nighttime).

D. NCSE induction via intra-hippocampal KA injection

NCSE seizure induction was performed on two consecutive days at P43-P44. Male peri-adolescent Sprague-Dawley rats received two 24-hour apart intra-hippocampal injections of either KA or normal saline (the vehicle) as follows;

RKA group: two episodes of NCSE were induced by two 24-hours apart intra-hippocampal KA injections, 0.00625 μg KA each, dissolved in normal saline, a sub convulsive dose which results in an electrographic pattern and behavioral manifestations comparable to those seen with NCSE of temporal lobe origin.

SKA group: one episode of NCSE was induced by an intra-hippocampal injection of KA (0.00625 μg in normal saline). This group received an intra-hippocampal injection of normal saline on the second day of induction.

Control group: sham manipulation was performed by injecting two 24-hour apart doses of normal saline (the vehicle) to account for volume induced mechanical excitation, if any.

Behavioral signs and EEG continuous tracing of all rats were monitored for two hours after each injection followed by long-term video-EEG recording.

E. Cognitive and emotional behavioral panels

Rats belonging to the three groups were subjected to a full battery of cognitive and behavioral panels, starting one day post the second induction day (at P45). These tests were tailored to test amygdalo-hippocampal functions, and performed in a sequence from the least aversive to the most in the following order: Light-Dark box test (LDT), Open Field test (OFT), Forced Swim test (FST), Morris Water Maze test (MWM), and lastly the Modified Active-Avoidance test (MAAV). (Testing is still ongoing to reach a power of 15-20 rats per group)

1. The light-dark box test (LDT)

The test was conducted over a single 5-minute session in which rats were allowed to freely explore a novel environment composed of two different compartments using a shuttle box described below (see section III-E-5). One of the compartments (left) is brightly lit with white walls and supplemented with visual cues (dice, beads, …etc), while the other (right) is kept dark and surrounded by black walls and no visual cues (see figure 12). The test was initiated by placing the rat into the dark compartment. The apparatus was cleaned between animals with an unscented detergent followed by 70% ethanol. The total time spent in each compartment as well as the number of transitions between the two compartments were recorded using Graphic State 4 software (Coulbourn Instruments, Harvard Apparatus, USA).

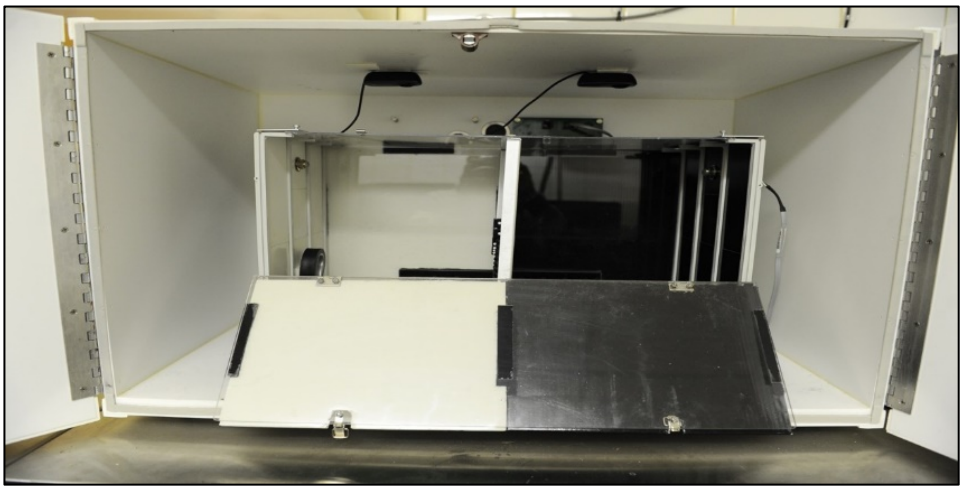

Figure 12 SETUP OF THE LIGHT-DARK BOX
The left compartment has white walls with visual cues (not seen in this image). The right compartment has black walls with no visual cues.

2. The open field test (OFT)

The OFT was conducted over three consecutive days. Each day, rats were allowed to freely roam for three minutes in an opaque plexiglass square field apparatus (W 80 cm, L 80 cm, H 40 cm) (see figure 13). On the first session, a small object (cube, dice or a bottle) was placed in the center of the field's floor and rats were placed in the nearest corner to the object then allowed to freely explore their surroundings. On each of the two subsequent days, a novel object was added, and rats' exploration behaviors were observed. The floor and walls of the field were wiped clean between animals with an unscented detergent followed by a 70% alcohol solution. The motion track of each rat throughout sessions was video recorded then analyzed using the Panlab SMART video tracking 3.0 software.

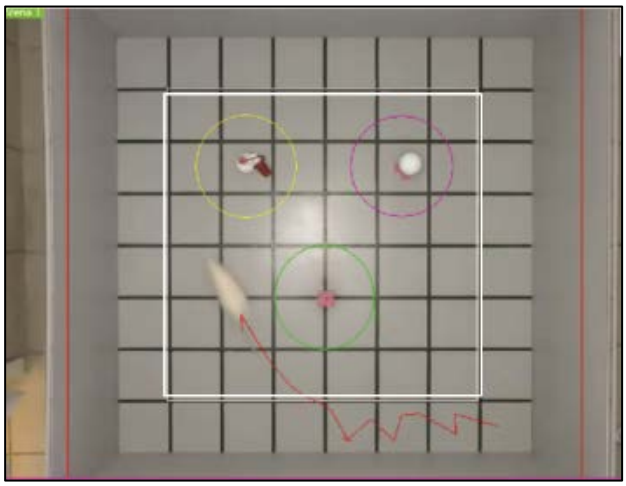

Figure 13 OPEN FIELD TEST APPARATUS
The rat is placed into the nearest corner to the novel object and left to freely explore its surroundings for 3 minutes. Shown here is a session on the third day with 3 objects (ball, cube, and bottle) in place.

3. The forced swim test (FST)

The test consisted of two 24-hours apart 10 minutes sessions. A clear transparent plexiglass cylinder (height 50 cm, diameter 20 cm) filled with water to a height of 35 cm from the base was used for this test (see figure 14). Rats were place in water and left to swim while being video recorded. Their swimming behaviors were analyzed using the SMART software (Panlab, Harvard apparatus, USA) by calculating the length of time animals spent immobile, the time of active swimming, and the number of climbing attempts. Mobility was defined as any movements other than those necessary to balance the body and keep the head above the water (floating). Water was changed after each animal, and rats were allowed to dry under a heat lamp in a cage covered with an absorbent towel.

Figure 14 FORCED SWIM TEST SETTINGS
Rats were put into a water filled cylinder and left swimming for 10 minutes. A Camera was used to video record their swimming behaviors for later analysis.

4. The Morris Water Maze test (MWM)

The test was conducted in a large dark- blue circular pool (150 cm in diameter and 80 cm in height) filled with water (25 °C) to a depth of 30 cm (see figure 15). The pool was divided into 4 quadrants by two imaginary perpendicular lines with the surrounding walls being supplemented with visual cues that were kept constant throughout training and testing days. On the first day, each rat was allowed to freely swim in the pool for two minutes, without a rescue platform, in order to habituate animals to the testing environment. Later the same day, an invisible escape platform was placed 2 cm below water surface in one quadrant of the pool and remained there for the rest of learning acquisition days (days 1-5). For five days, rats were subjected to four training trials a day to swim and reach the platform with a 30 second resting period between trials. Rats were immersed in water facing the pool wall and given two minutes to find the platform, however, whenever they fail to reach it, the operator will place the rat on the platform for 30 seconds. Each training day, rats' immersion point was changed. On day 6, a probe trial was performed to asses retention of spatial navigation

memory. The platform was removed, and rats were immersed into water with a novel starting point in the quadrant opposite to where the platform was located on training days. Rats were given two minutes to swim freely in the pool with their track of motion and time spent in each quadrant being recorded. After the probe trial, on the same day, rats' motor and visual capabilities were assessed by placing a visible platform in the pool and allowing rats to swim into it for four trials per rats. The latency period from immersion in the pool to reaching the platform was recorded. All test trials were video-recorded and analyzed using the automated SMART software (Panlab, Harvard apparatus, USA).

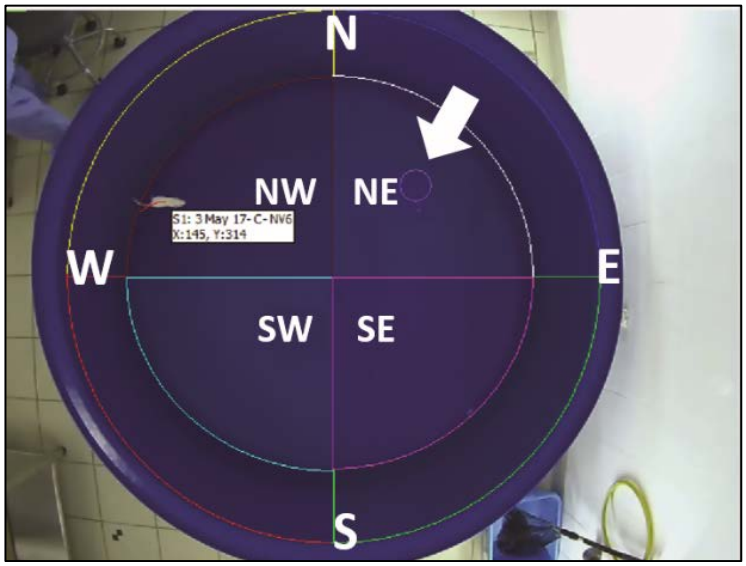

Figure 15 THE MORRIS WATER MAZE TRAINING SESSION
The rat is placed into the maze and allowed to swim to find an invisible platform (white arrow). NE: north-east; SE: south-east; SW: south-west; NW: north-west.

5. The Modified active-avoidance test (MAAV)

To investigate the late effects of NCSE episodes on the integrity of the amygdalo-hippocampal circuitry and the ability to learn contextual and auditory cued adaptive shock avoiding behaviors, the modified active-avoidance test (MAAV), a test

developed at our laboratory, was performed at P45 (one day after the second KA injection) for the short term paradigm and at P84 for the long term paradigm. The test was conducted in a standard shuttle box (Coulbourn Instruments, USA), consisting of two equal compartments (H 34 cm, W 27 cm, L 27 cm), connected via a 9x9 cm opening located in the middle of the metallic partition wall. The box is placed in a soundproof isolation cubicle (H 80 cm, W 53 cm, L 53 cm) (Coulbourn Instruments, USA). It is also equipped with a tone generator (auditory cue, 4 kHz, 86 dB) and infrared beam sensors that detect transitions between the chambers. Videos in both chambers were recorded for later interpretation (see figure 16).

Figure 16 THE MODIFIED ACTIVE-AVOIDANCE TEST SHUTTLE BOX SETUP
A standard shuttle box placed in a sound-proof isolation cubicle and equipped with a tone-generator, infrared beam sensors, and two video recording cameras fixed on the cubicle ceiling above the center of each compartment. The left compartment walls are covered with white foam panels whereas the right one is covered with black and white striped foam and augmented with visual cues (such as dices and beads) for contextual conditioning.

The MAAV test consisted of one day of habituation (Day 0) followed by six days of shock avoidance training (Days 1-6), and one final day of retention testing (Day 7). On habituation day, rats were allowed to freely explore the shuttle box compartments for a period of 5 minutes to adapt to the novel environment without any threats or cues. During training days, a protocol is customized using the Graphic state 4

software so that an incoming electrical foot shock (0.5 mA, 15-second duration) is signaled with a 15 second tone in the left compartment (40-second inter-trial rest period on that side) but not in the right one, where an electrical foot shock is delivered every 10 seconds spent in that compartment. Here, compared to the habituation day, the left chamber remains unchanged, but the right chamber is modified (foam plate with black-white strip patterns and visual cues such as dices and beads) for contextual conditioning training (see Figure 17). Shuttling through the opening between the two compartments prevents an incoming shock (shock avoidance) or terminates an ongoing one (escape). Cycles in both the left and right chambers are repeated for a total of 30 signaled trials. After training, a two-part retention test was performed on day 7. The first part consisted of two sessions, two minutes each, where the rat was allowed to freely roam in the shuttle box without tone stimuli or shocks in order to assess retention of contextual learning (the compartments pattern remains as they were on days 1-6). In the second part of the retention subtest, the visual cues were removed from the right chamber and 30 tone-signaled avoidance trials were delivered in either compartments with an inter-trial interval of 30 seconds. The 30 avoidance trials were followed by two minutes of continuous tone to assess freezing responses. Chambers were cleaned with unscented detergent then 70% alcohol solution after each rat. The rats' movements were video recorded then analyzed using SMART video tracking 3.0 software (Panlab, Harvard apparatus, USA).

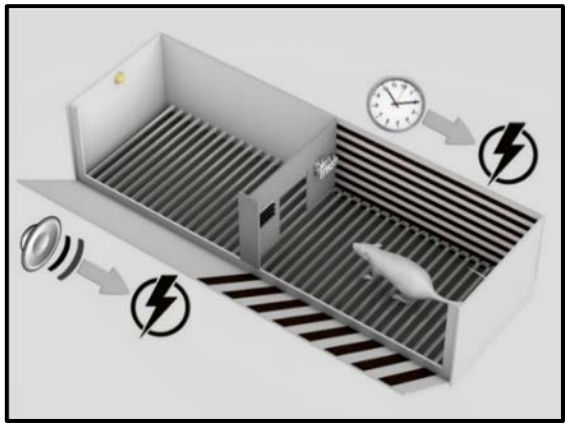

Figure 17 SCHEMATIC DESIGN OF THE MODIFIED ACTIVE AVOIDANCE TEST (MAAV)
Developed in our laboratory; this test utilizes a modified shuttling box designed to simultaneously test conditioning to both contextual and auditory stimuli and to assess learning of hippocampal-dependent adaptive shock-avoiding behaviors in rodents. Shocks are delivered following a tone signal in the left compartment unless the rat shuttles through the opening to the context-filled right compartment where shocks are delivered after each 10 seconds spent in it without shuttling.

F. Transcardial perfusion surgery

All rats underwent transcardial perfusion non-survival surgery 30-90 minutes after the MAAV retention test (at P52 in the short-term paradigm and at P91 in the long-term paradigm) to ensure the detection of learning-induced freshly expressed plasticity marker protein (Syp). Rats were anesthetized with an intraperitoneal injection of a Ketamine/Xylazine cocktail (Ketamine 80 mg/kg, Xylazine 10 mg/kg). Once deep anesthesia was achieved (confirmed by unresponsiveness to noxious stimuli), the animal was pinned to a tray under a chemical fume hood with the chest upward and a midline incision was made to open the abdominal cavity. The animal's heart was exposed by cutting the diaphragm and the ribs along the lateral surface. A custom perfusion cannula was inserted into the left ventricle and a cut in the right atrium was immediately made to allow drainage. The animal was then perfused with 100 ml of 0.1 M phosphate-buffered saline (PBS) (1X) solution flush blood from the circulatory

system, followed by 100 ml of 4% depolymerized paraformaldehyde in phosphate buffer (pH =7.4).

After decapitation, brains were harvested, post-fixed for 48 hours in 4% PFA solution and finally were stored in a 30% Sucrose solution at 4°C for later paraffin embedding, sectioning and histological analyses.

G. Histological studies

Coronal sections of 8 µm in thickness were obtained for histological and immunohistochemical analyses. Sections were selected by visual inspection to match the structural pattern of coronal sections located 2.4 – 2.7 mm posterior to the Bregma in the Sherwood and Timiras atlas of the developing rat brain. (156) This histological landmark was chosen based on the assumption that if any injury exists, it is expected to be most detectable near the site of KA injection.

1. Immunohistochemistry

Immunohistochemistry was performed on brain sections of a sample of 3-4 rats from each group. Slides were first deparaffinized with xylene and rehydrated through a series of descending grades of alcohol solutions. Slides were incubated for 60 minutes in 90 °C sodium citrate buffer (10 mM, pH = 6) for antigen retrieval and then treated for five min at room temperature with 3% H_2O_2 to neutralize endogenous peroxidase. Slides were incubated overnight at 4 °C with primary antibody solutions of the following antibodies: anti-GFAP (MCA-5C10; dilution 1:1000, EnCor Biotechnology), anti-NeuN (MAB377; dilution 1:100, Millipore), and anti-Syp (sc-12737; dilution 1:2000, Santa Cruz Biotechnology). Sections were then incubated with peroxidase

conjugated anti mouse secondary antibody for one hour at room temperature (Leica Biosystems, UK). Immunohistochemical staining was visualized under the light microscope following application of 3,3'-Diaminobenzidine (DAB) and Hematoxylin counterstaining. The average neuronal density and the optical density of GFAP and Syp stained sections were calculated by an investigator blinded to treatment groups using ImageJ (NIH, USA).

2. Image analyses

Immunostained brain sections were imaged using the uSCOPE (uScope MXII, USA) machine. Rats' hippocampi were divided for histological analyses into 3 areas of interest each. The CA1-subiculum and CA2-CA3 areas were determined by extending an imaginary line from the tip of the dentate gyrus to the distal border of the hippocampus passing through the tip of the dentate inner blade. Dentate hilar zone border was determined by a line extending from the tip of the inner blade to the outer blade's border (see figure 18). CA1-CA3 NeuN positive cells were counted manually (6 sections per brain, 3 brains per group) by an investigator blinded to treatment groups. Hippocampal optical density was measured on GFAP and Syp stained sections (3 sections per brain, 3 brains per group for each antibody) using ImageJ software (NIH, US). The surface area of the hippocampus was also measured after manually outlining its borders along the ventricles ventromedially, and the corpus callosum dorsolaterally with the same software.

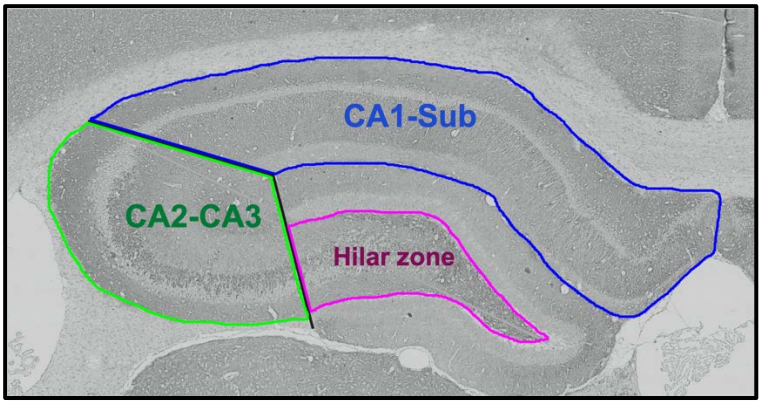

Figure 18 HIPPOCAMPAL AREAS DIVISION FOR ANALYSES
Rat's dorsal hippocampus was divided into three areas of interest: CA1-subiculum (blue), CA2-CA3 (green), and dentate hilar zone (purple) to analyze for potential neuronal loss with NeuN staining, reactive astrogliosis with GFAP levels, and alterations in hippocampal synaptic plasticity with Syp staining.

H. Statistical analyses

All data analyses were performed using Prism 8 (GraphPad Software, USA). Animals were randomized to treatment groups prior to seizure induction or any data analysis. Unless otherwise stated, data are presented in graphs as mean ± standard error of the mean (SEM). The MAAV learning acquisition, FST, and the MWM spatial acquisition data were analyzed using two-way analysis of variance (ANOVA) with repeated measures followed by post hoc Fisher least significant difference (LSD) test, whereas MAAV retention, LDT, and OFT data were analyzed using one-way ANOVA with post hoc Fisher LSD test. Histological densitometric measurements and cell count analyses were performed using NIH ImageJ software. A p-value of less than 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

A. Intra-hippocampal KA injections and patterns of electrographic NCSE

Subconvulsive KA doses administered at the first injection day to the SKA group and both injection days to the RKA group induced prolonged non-convulsive electrographic seizures, in all rats, accompanied by behavioral changes. These behavioral changes included oromotor automatisms, behavioral arrest (motionless persistent staring), and unresponsiveness to intermittent tapping on the cage, strictly corresponding to stages 1 and 2 of the Racine scale (140). Electrographic seizures consisted of rhythmic fast spikes and spike waves with occasional polyspikes (see figure 19). The latency to seizure onset on the first injection day was comparable between the SKA group and RKA group (15.74 ± 3.30 for SKA, 19.83 ± 3.55 minutes for RKA, $p>0.05$, Student's t test). The duration of seizures was also comparable between the two groups (84.59 ± 7.78 minutes for SKA, 72.99 ± 9.74 minutes for RKA, $p>0.05$, Student's t test) (see figure 20). On the second injection day, the latency to seizure onset in the RKA group (19.89 ± 1.92 minutes) and the duration of seizures (50.68 ± 9.76 minutes) were comparable to those of the first injection day ($p>0.05$, Student's t test). Exclusion criteria included a seizure activity lasting less than 30 minutes or lasting more than 180 minutes, the failure to induce an electrographic seizure activity after the administration of a KA injection (no seizure onset 60 minutes after the chemoconvulsant injection), or the emergence of electrographic seizure activity after a saline injection.

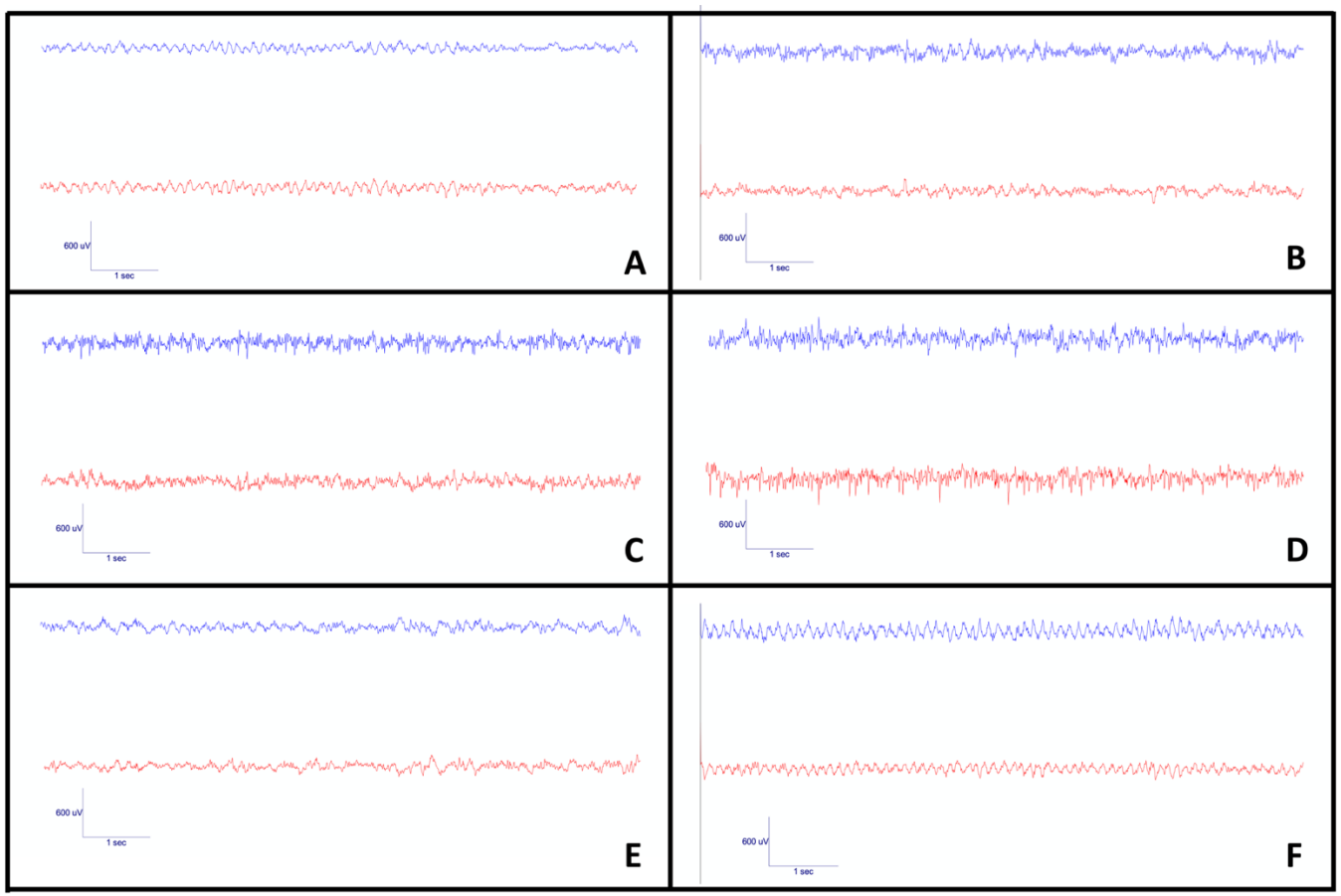

Figure 19 EEG PATTERNS OF NCSE EVOLUTION SHOWN IN LONGITUDINAL BIPOLAR MONTAGE

Shown is electrographic EEG tracing of a rat with four sampling electrodes (F3: left frontal, F4: right frontal, P3: left parietal, and P4: right parietal) in a longitudinal bipolar montage with a notch set at 50 Hz. **Panel A.** Baseline (4-6 Hz) electrographic brain activity of an awake rat. **Panel B.** Focal NCSE onset after intrahippocampal Kainic acid (KA) injection. The tracing shows bilateral fast activity more prominently seen on the left side (Blue; F3-P3) with recognizable spikes and spike waves. **Panel C.** Generalization of NCSE (being spread to the right side of the brain contralateral to injection site). **Panel D.** NCSE offset characterized by a theta activity intermixed with slow waves. **Panel E.** and **F.** Complete NCSE offset; return to baseline 6 Hz electrical activity.

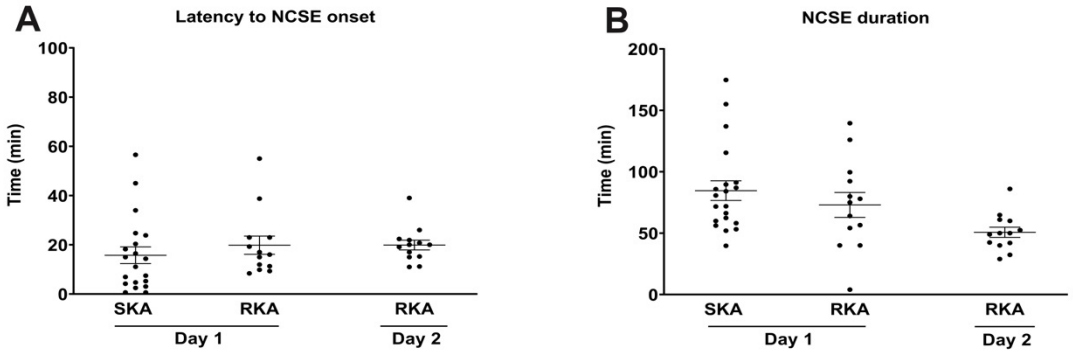

Figure 20 INDUCED SEIZURE CHARACTERISTICS

Panel A. The average latency to NCSE onset post-KA injections was comparable between the SKA group (15.74 ± 3.30 minutes, n=20) and the RKA group (19.83 ± 3.55 min on day 1, and 19.89 ± 1.92 min on day 2, n=13). **Panel B.** The duration of electrographic seizures was on average 84.59 ± 7.78 min in the SKA group, and 72.99 ± 9.74 minutes on day 1 and 50.68 ± 9.76 minutes on day 2 in the RKA group. None of controls developed any seizure activity. Results reported as mean + SEM. (CTR: controls, SKA: single kainic acid injection, n=20; RKA: repeated kainic acid injections, n=13)

Furthermore, to investigate potential NCSE-induced electrographic changes we prolonged the EEG recording in a cohort of rats (n=5 per group) in the long-term paradigm until the age of P72. No spontaneous recurrent seizures were detected during the 4 weeks post-NCSE induction. Nonetheless, both SKA and RKA groups had a higher frequency of spikes and polyspikes observed on EEG tracings with a gradual drop in spike frequency over time (see figure 21).

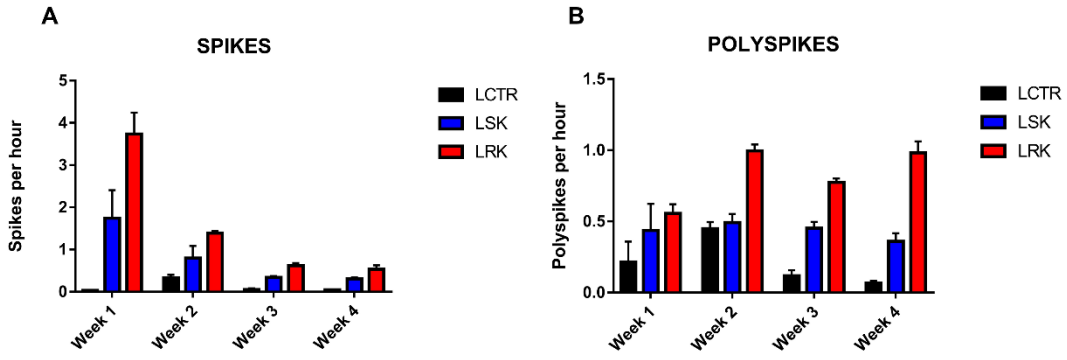

Figure 21 ANALYSES OF THE CONTINUOUS ELECTROENCEPHALOGRAM (EEG) TRACINGS

Spikes and polyspikes were quantified by sampling 8 hours per day; 4 hours from the daytime and 4 hours from the nighttime. **Panel A.** Both the SKA and RKA groups had a higher frequency of spikes compared to controls, which had none, with the RKA group showing slightly higher numbers. These newly emerged spikes gradually decreased in frequency over time almost returning to baseline. **Panel B.** Both SKA and RKA groups had an increased frequency of polyspike components compared to controls

following kainic acid injections with more increase in the RKA group. Results reported as mean + SEM. (CTR: Controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n=5 per group)

B. Early effects of NCSE on contextual and auditory learning

The MAAV test was used to investigate the effect of one or two episodes of temporal lobe NCSE on the amygdalo-hippocampal function, specifically emotional-relevant learning and adaptive avoidance to aversive stimuli. To confirm preliminary data previously obtained in our lab, we increased the number of animals in each group to reach a number of n=15-20 in order to be sufficiently powered to establish statistical significance. During the 6 days of auditory learning acquisition, rats of all groups eventually learned to avoid tone-signaled foot-shocks in the left compartment (see figure 22.A). However, when compared to controls both the SKA and RKA groups had a significant drop in avoidance rates (on day 5 for SKA, on days 3 and 5 for RKA, $p<0.05$, two-way ANOVA). Retention of auditory learning on the last day of testing was comparable between controls and the SKA group, nevertheless, a still remaining deficit was observed in the RKA group ($p<0.05$, one-way ANOVA) (see figure 22.B). In the acquisition of context-cued shock-avoidance, the performance of SKA rats was comparable to controls, on the other hand, RKA rats were slower to learn than controls with a statistically significant difference on day 4 ($p<0.05$, two-way ANOVA). Interestingly, SKA rats had significantly higher avoidance rates than RKAs on days 2-5 ($p<0.05$, two-way ANOVA) (see figure 22.C). Moreover, although the latency to exit the right compartment during contextual retention testing of both SKA and RKA groups was comparable to controls (probably caused by high variability between animals), SKA rats were significantly faster than RKA rats ($p<0.05$, one-way ANOVA). (see figure 22.D).

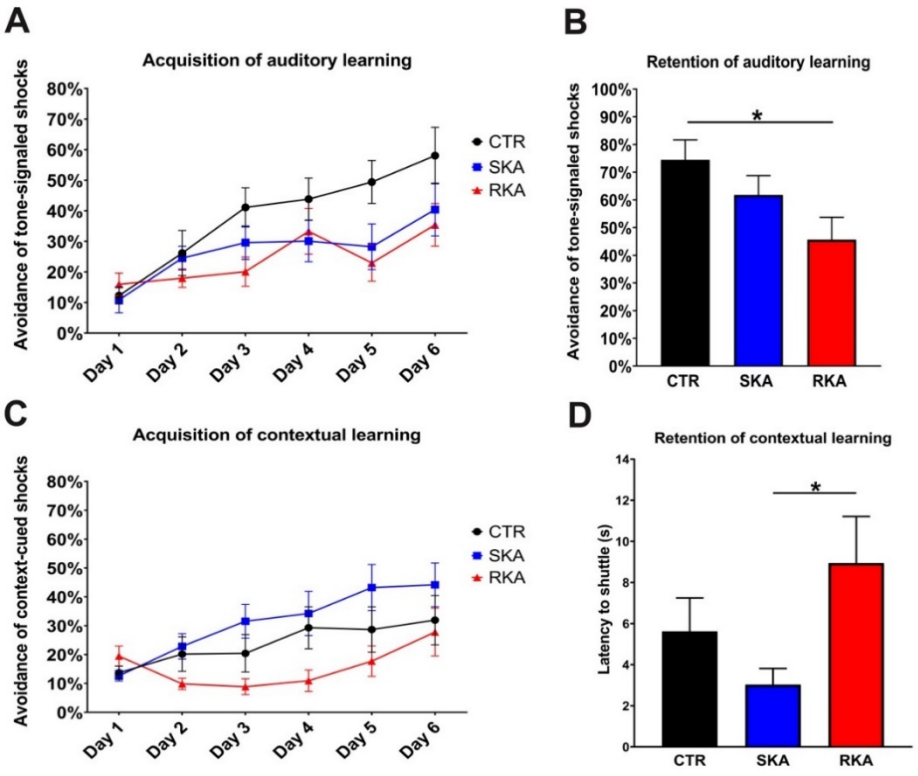

Figure 22 EARLY LEARNING DEFICITS IN THE MODIFIED ACTIVE AVOIDANCE (MAAV) TEST

Panel A. A curve representing the learning acquisition of tone-signaled foot-shock avoidance during the 6 days of training. RKA group had significantly lower avoidance rates compared to controls on days 3, 5, and 6 ($p<0.05$, two-way ANOVA). SKA group had a lower percentage of shock avoidance than controls only on day 5 ($p<0.05$, two-way ANOVA). **Panel B.** Auditory retention testing revealed a learning deficit in RKA group with statistically significant lower avoidance rates compared to controls ($p<0.05$, one-way ANOVA), while SKA rats retention rates were comparable to those of controls ($p>0.05$, one-way ANOVA). **Panel C.** a curve representing the learning acquisition of context-cued foot-shock avoidance during the 6 days of training. RKA group avoidance rates did not increase with time unlike other groups and were significantly lower than SKA group on days 2,3,4, and 5, and lower than controls on day 4 (p,0.05, two-way ANOVA). The SKA group showed a trend of enhanced learning acquisition compared to controls, but it did not reach statistical significance. **Panel D.** In the contextual learning retention subset, the SKA group had the shortest latency (3.0 ± 0.8 s) to exit the right compartment the reached statistical significance when compared to the RKA group (8.9 ± 2.3 s, $p<0.05$), but not to the control group (5.6 ± 1.6 s, $p>0.05$, one-way ANOVA). results reported as mean + SEM. (CTR: controls, n=18; SKA: single kainic acid injection, n=23; RKA: repeated kainic acid injections, n=22)

C. Histological and structural analyses

1. Hippocampal neuronal density: Neuronal nuclear antigen (NeuN)

To investigate potential mechanisms underlying NCSE-induced deficits in hippocampal-dependent learning, we assessed hippocampal pyramidal neuronal

densities of NeuN-stained sections. NeuN is generally considered a marker of mature neuronal cells. The number of neurons in CA1-CA3 subfields of both the left and right hippocampi were quantified and normalized by the surface area. Rats belonging to all three groups had comparable pyramidal neuronal densities in the left hippocampus where KA injections were administered ($p>0.05$, One-way ANOVA) (see Figure 23). Similarly, no statistically significant difference in pyramidal cell densities was detected in the right hippocampus ($p>0.05$, one-way ANOVA). These results indicate the absence of overt hippocampal neuronal loss resulting from neither the chemical effect of the administered sub-convulsive doses of KA nor the herein induced NCSE episodes.

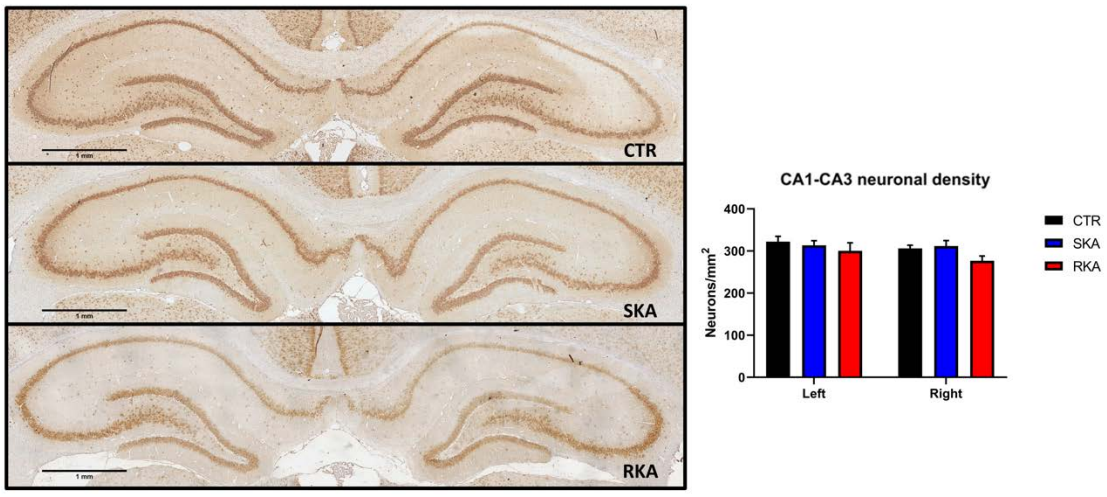

Figure 23 HIPPOCAMPAL PYRAMIDAL NEURONAL DENSITY
Neuronal densities were calculated by dividing the number of NeuN-positve cells in the CA1-CA3 regions over the hippocampal surface area. Hippocampal neuronal densities on both left and right sides were statistically comparable ($p>0.05$, one-way ANOVA) among all groups (left 300.5 ± 18.9 neurons/mm^2, and right 276.7 ± 11.5 neurons/mm^2 for RKA; left 313.5 ± 11.0 neurons/mm^2, and right 311.7 ± 13.3 neurons/mm^2 for SKA; left 322.2 ± 12.5 neurons/mm^2, and right 306.0 ± 7.9 neurons/mm^2 for controls). Reported as means ± SEM (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n= 3 brains per group, 6 sections per brain)

2. Reactive astrogliosis: Glial fibrillary acidic protein (GFAP)

To further assess potential NCSE related hippocampal molecular and morphological changes, GFAP immunostaining was performed on brain sections of all groups to test for reactive astrogliosis. The intensity of GFAP expression was detected as a value of optical density of GFAP-positive astrocytes in CA1, CA2-CA3, and the dentate hilar zone regions of the left and right hippocampi. In the CA1 region, there was no statistically significant difference in GFAP expression among experimental groups ($p>0.05$, One-way ANOVA), however, GFAP levels in both CA2-CA3 region and the hilar zone were significantly higher in groups of rats that underwent NCSE seizures (SKAs and RKAs) compared with sham manipulated controls ($p<0.05$, One-way ANOVA) (see figure 24). This increase in expression was apparent in both sides of the hippocampus (ipsilateral and contralateral to the KA injection) supporting the assumption that NCSE in itself can cause bilateral reactive astrogliosis in the hippocampus.

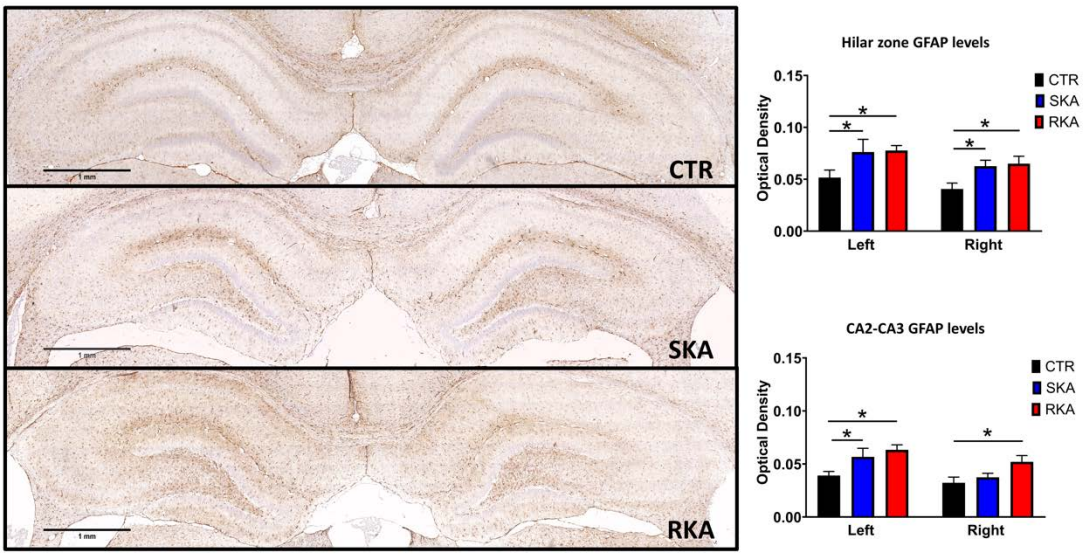

Figure 24 HIPPOCAMPAL GFAP EXPRESSION PATTERNS

The optical density of GFAP-positive astrocytes was significantly higher ($p<0.05$, One-way ANOVA) in groups that underwent one or two episodes of NCSE in hippocampal CA2-CA3 (Left 0.063±0.005, and right 0.052±0.006 for RKA; Left 0.057±0.008, and right 0.037±0.004 for SKA) and dentate hilar zone (Left 0.078±0.005, and right 0.065±0.007 for RKA; Left 0.076±0.012, and right 0.063±0.006 for SKA) regions compared with sham manipulated controls (Left 0.039±0.004, and right 0.032±0.005 for CA2-CA3; Left 0.052±0.007, right 0.041±0.006 for Hilar zone), except for the CA2-CA3 region of the right SKA hippocampi where the difference between SKA and controls did not reach statistical significance ($p>0.05$, One-way ANOVA). Reported as mean ± SEM (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n= 3 brains per group, 3 sections per brain)

3. Synaptic plasticity: Synaptophysin (Syp)

To investigate potential NCSE-induced alterations in hippocampal synaptic plasticity, Syp protein levels were examined in brain sections of all study groups post-MAAV testing. Syp reactivity is expected to appear in the neuropil. In the CA1 region, there was no statistically significant difference in Syp expression among all experimental groups ($p>0.05$, One-way ANOVA). However, while SKA and control groups had comparable levels of hippocampal Syp protein expression ($p>0.05$, one-way ANOVA), RKA rats had significantly lower Syp levels in the supra-pyramidal and infra-pyramidal bundles of the CA2-CA3 subfields of the hippocampus and the dentate hilar zone when compared with SKA and control rats ($p< 0.05$, one-way ANOVA) (see figure 25). In other words, two episodes of temporal lobe NCSE resulted in an increase in hippocampal synaptic density along the dentate-CA3 mossy fiber pathway.

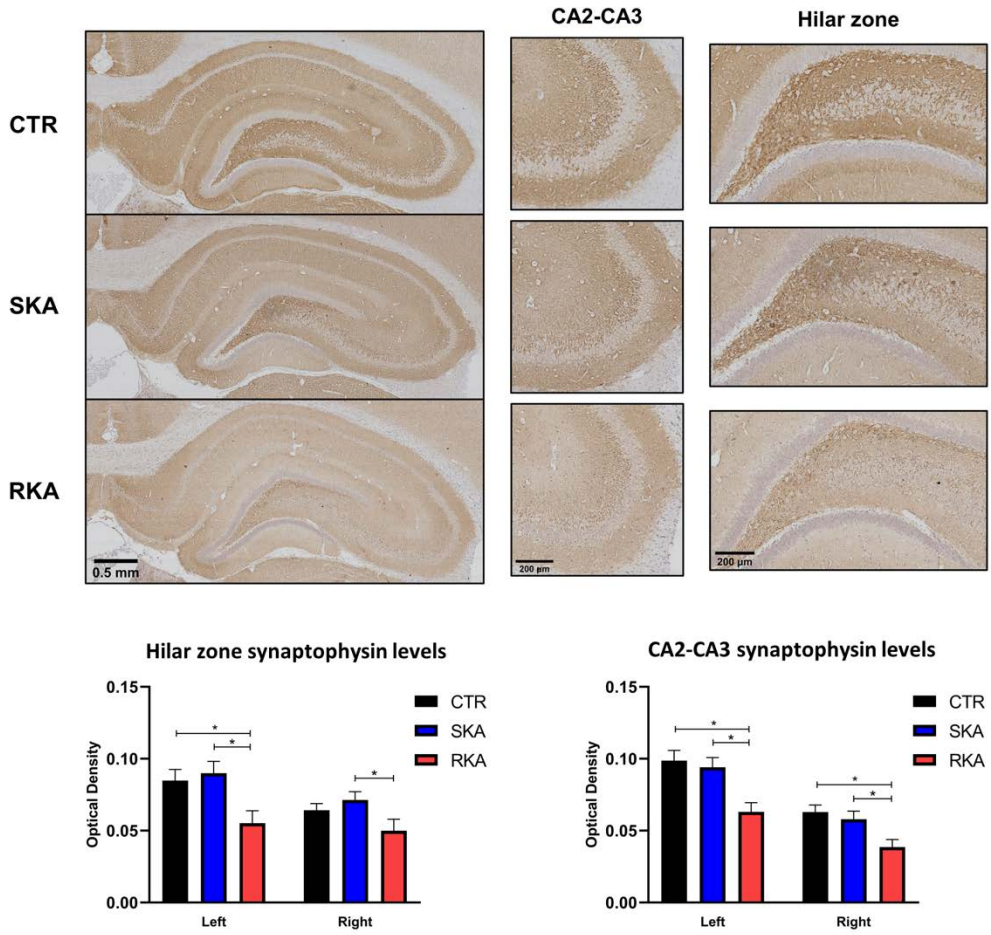

Figure 25 ALTERATIONS IN HIPPOCAMPAL SYNAPTIC PLASTICITY MARKER, SYNAPTOPHYIN

Shown is a light photomicrograph of sections belonging to all three experimental groups. decreased levels of Synaptophysin expression in the RKA group compared with the SKA and control groups can be observed by visual inspection of the suprapyramidal and infrapyramidal regions of CA2-CA3 subfield and the dentate hilar zone. Optical density measurements of Synaptophysin levels in the CA2-CA3 area of both left and right hippocampi revealed a significant decline in Synaptophysin protein expression in the RKA group (Left CA2-3 0.063±0.006, Right CA2-3 0.039±0.005) compared with the SKA (Left CA2-3 0.094±0.007, Right CA2-3 0.058±0.006) and control (Left CA2-3 0.099±0.007, Right CA2-3 0.063±0.005) groups. Similarly, Synaptophysin protein expression levels measured by optical density in the dentate hilar zone were significantly decreased in the left hippocampi of RKA group (0.055±0.009) when compared with both SKA (0.09±0.008) and control (0.085±0.008) groups, however, the decrease in the right hippocampus was significant in RKA (0.05±0.008) when compared with SKA group (0.071±0.006), but not with controls (0.064±0.005).

D. Later life effects of NCSE on cognitive and behavioral functions

1. Exploratory and anxiety-like behaviors in the LDT and OFT

We investigated the late effects of one versus two NCSE episodes on activity levels, exploratory tendencies, and anxiety-like behaviors in closed (LDT) and open (OFT) novel environments. In the LDT, both SKA and RKA groups had a trend of decreased percentage of time spent in the lit compartment of the box compared with controls, more so in the RKA group (see figure 27). The number of entries into the lit compartment were comparable among all groups (5 ± 0.89 for CTR, 6.4 ± 0.81 for SKA, 4.4 ± 1.29 for RKA). This preliminary data set hints to a possible increase in anxiety-like behaviors with a decrease in exploratory tendencies following two episodes of NCSE.

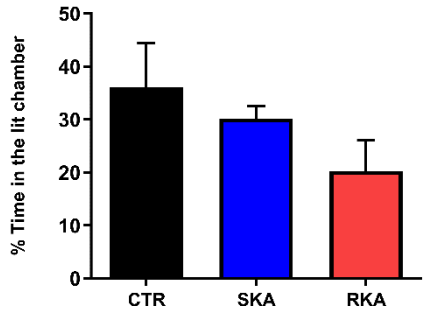

Figure 26 LATER LIFE ANXIETY-LIKE BEHAVIORS IN THE LIGHT-DARK BOX TEST

Statistics were not performed but a trend of decrease in the percentage of time spent in the lit compartment was observed with increased number of NCSE episodes (36.15% ± 8.25 for CTR, 30.17% ± 2.40 for SKA, 20.33% ±5.822 for RKA). Reported as mean ± SEM (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n=5 per group)

In the OFT, all groups spent comparable durations in the arena's periphery and in object exploration on each testing session. However, there was a trend of increase in the cumulative total distance traveled by SKA and RKA rats over the three testing

sessions compared with controls (see figure 28). This increase point to NCSE-induced hyperactivity if proven statistically significant in our ongoing studies.

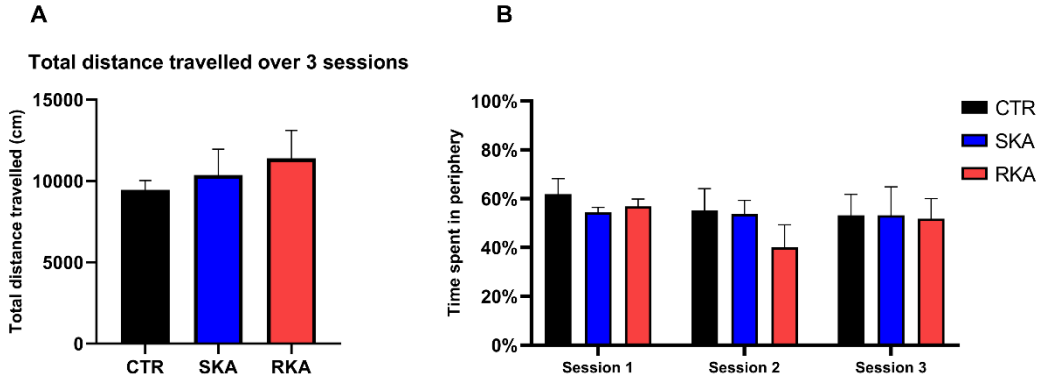

Figure 27 LATER LIFE EXPLORATORY BEHAVIORS AND HYPERACTIVITY IN THE OPEN FIELD TEST

Panel A. Statistics were not performed but a trend of increase in the total distance traveled in the open field during all three sessions of testing was observed with increased number of NCSE episodes (9462 ± 573 cm for CTR, 10363 ± 1595 cm for SKA, 11390 ± 1719 cm for RKA). **Panel B.** The percentage of time spent in the peripheral zone of the open field was comparable among all groups in all three sessions. Reported as mean ± SEM (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n=5 per group)

2. Depressive-like behaviors in the FST

The FST test is used to assess depressive-like behaviors by scoring active (swimming and climbing) and passive (immobility) movements of rodents when forced to swim in a cylinder from which there is no escape. Here, we assessed the delayed effects of one or two episodes of NCSE on rats' performance in the FST. In this preliminary data set, the percentage of immobility during the 10 minutes of testing showed a trend of increased immobility on the first day of testing in groups that underwent NCSE episodes (SKA and RKA) when compared to controls (see figure 29.A). However, on the second day of testing, all groups had comparable immobility percentages (see figure 29.B).

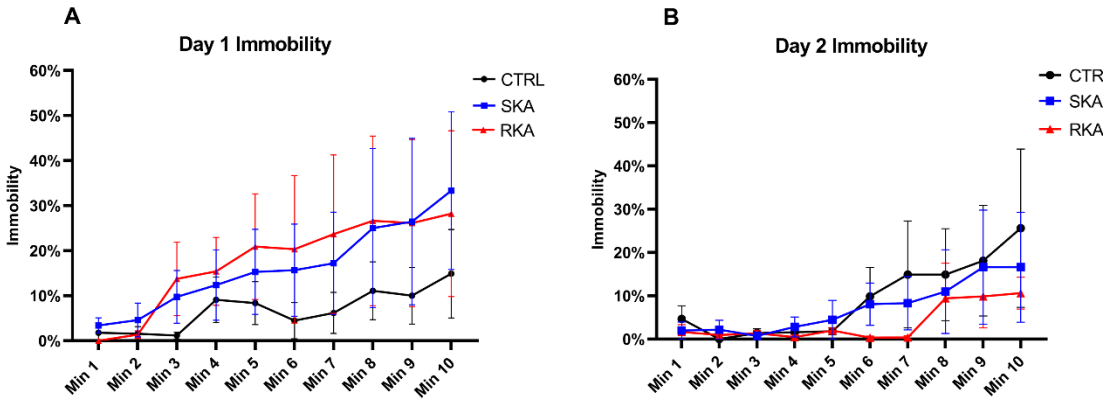

Figure 28 LATER LIFE DEPRESSIVE-LIKE BEHAVIORS IN THE FORCED-SWIM TEST

Panel A. During the 10 minutes of testing on both days, both the SKA and RKA groups exhibited decreased struggling behaviors throughout the 10 minutes of first day testing and were trending toward an increase in immobility percentages. **Panel B.** Immobility percentages on the second day of testing were comparable between all groups. Reported as means (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n=5 per group)

3. Visuospatial Navigation in the MWM

In order to evaluate NCSE late effects on visuospatial learning, rats were subjected to the MWM test. During the 5 days training trials, the escape latencies of control and SKA groups were comparable, however, rats of the RKA group were slower in learning to escape and reach the platform (see figure 30). This preliminary data (n=5 per group) point to a potential delayed effect of NCSE on hippocampal-dependent contextual memory and visuospatial navigation.

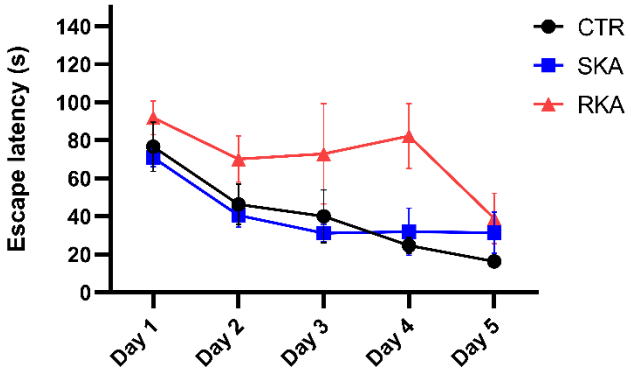

Figure 29 THE ACQUISITION OF VISUOSPATIAL NAVIGATIONAL MEMORY IN THE MORRIS WATER MAZE ONE MONTH POST-NCSE
In the 5 days of learning acquisition, while the RKA group had higher escape latencies to reach the platform throughout training days, the SKA and control groups showed comparable latency curves. Reported as mean ± SEM (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n=5 per group)

Additionally, in the probe trial on day 6, controls and SKA rats had a higher percentage of distance traveled in the NE quadrant of the water pool (where the platform was placed during training days) than the RKA group (see figure 31.A). Also, controls and SKA rats showed a trend of increased percentage of time spent in the NE quadrant, but not RKA rats (see figure 31.B). This may support the assumption of impaired visuospatial memory induced by recurrent episodes of NCSE. In the visible platform trial, RKA group showed a higher latency to reach the platform (see figure 31.C), however, while motor deficits are uncommon with NCSE, future studies will include Rotarod testing for motor function assessment.

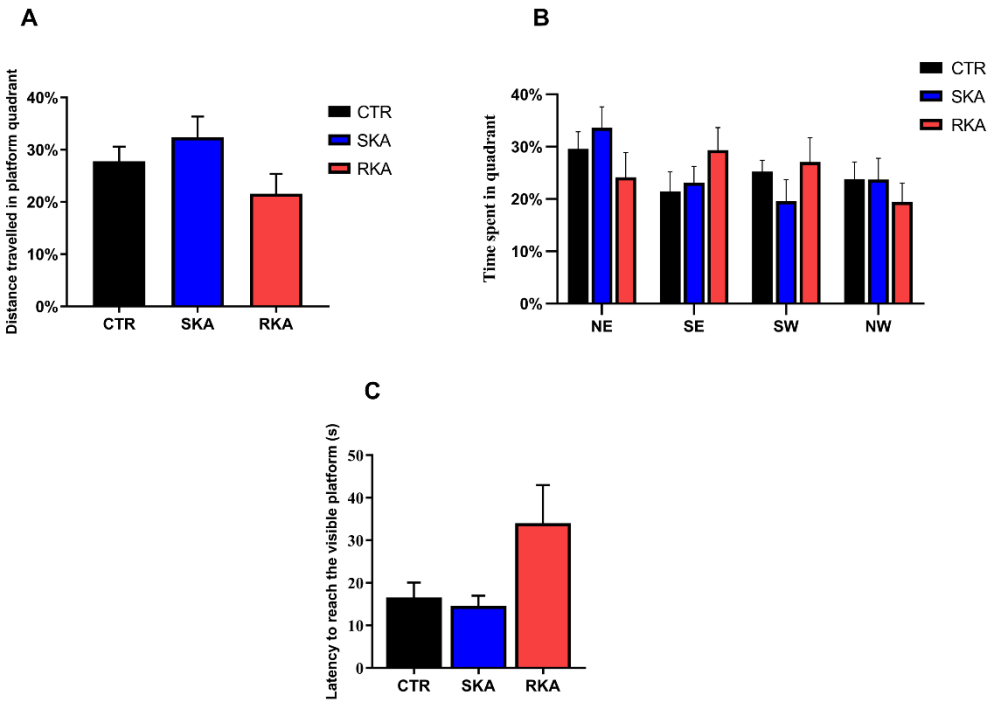

Figure 30 THE RETENTION OF VISUOSPATIAL NAVIGATIONAL MEMORY IN THE MORRIS WATER MAZE ONE MONTH POST-NCSE

Panel A. The percentage of total distance traveled during the probe trial in the NE quadrant (platform placement quadrant during training day) was slightly higher in controls and SKA rats than the RKA group (27.79 ± 2.79% for CTR, 32.37 ± 3.97% for SKA, 21.6 ± 3.78% for RKA). **Panel B.** Rats of both SKA and control groups spent a relatively higher percentage of time in the NE quadrant, however, the RKA group showed equal distribution of time spent between all water pool quadrants. **Panel C.** Latency to reach the visible platform was higher in the RKA (34.01 ± 8.971 sec) group compared with both SKA (14.60 ± 2.388 sec) and CTR (16.55 ± 3.492 sec). Reported as mean ± SEM (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n=5 per group)

4. Contextual and auditory learning in the MAAV

We subjected rats of all groups, in the long-term paradigm, to the MAAV test one month after NCSE induction by intrahippocampal KA injections. In this preliminary data set, during the 6 days of learning acquisition, rats of all groups learned to avoid tone-signaled foot-shocks in the left compartment. Controls and SKA groups showed comparable auditory-cued avoidance rates, however, RKA group seemed to have a higher learning curve than other groups (see figure 26.A). All groups had comparable rates of context-cued foot-shock avoidance, yet a drop in performance may

be noticed in the RKA group on days 4 and 5 (see figure 26.C). In addition, all three groups were comparable in auditory and contextual memory retention testing (see figures 26.B and 25.D).

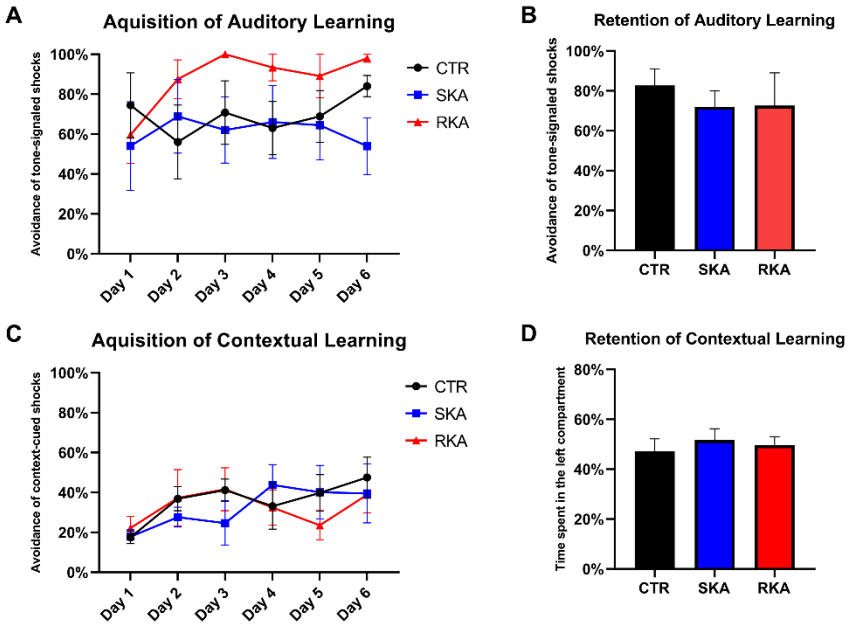

Figure 31 LATER LIFE CONTEXTUAL AND AUDITORY LEARNING IN THE MODIFIED ACTIVE AVOIDANCE (MAAV) TEST

Panel A. a curve representing the learning acquisition of tone-signaled foot-shock avoidance during the 6 days of training. RKA group had a relatively higher curve of learning when compared with both the SKA and control groups, which were comparable. **Panel B.** In auditory retention testing, all groups had comparable avoidance percentages (72.00±7.93 for SKA, 72.67±16.38 for RKA; 82.67±8.33 for CTR). **Panel C.** A curve representing the learning acquisition of context-cued foot-shock avoidance during the 6 days of training. All groups had comparable learning curves, yet a drop in RKA performance on days 4 and 5 might be observed. **Panel D.** In the contextual learning retention subset, all groups had comparable avoidance percentages (51.67±4.59 for SKA, 49.65±3.38 for RKA; 47.15±5.04 for CTR). Results reported as mean ± SEM. (CTR: controls; SKA: single kainic acid injection; RKA: repeated kainic acid injections; n=5 per group)

CHAPTER V

DISCUSSION

NCSE is a serious clinical condition that has been understudied compared with convulsive SE. Although a few recent reports have pointed to several cognitive and behavioral disturbances that can be attributed to NCSE (158, 159), the effects of this condition on the brain are still elusive due to insufficient knowledge. Here, we provide evidence for early life NCSE-related harmful effects on cognitive and emotional functions, in specific, emotionally-relevant learning and memory. Furthermore, we provide preliminary data for the potential persistence of these harmful effects in later life along with the emergence of hyperactive, depressive, and anxiety-like behaviors. We also report accompanying alterations in the hippocampal structural and molecular integrity evidenced by reactive astrogliosis and maladaptive changes in synaptic plasticity.

Our study revealed early post-NCSE learning deficits in periadolescence with later life persistence along with emerging psychiatric and emotional disturbances. Epilepsies are known to be associated with cognitive and psychiatric comorbidities that are debilitating especially in the pediatric population (160). Even though these comorbidities are well-described in the literature with CSE, we here report them following NCSE. We utilized a novel test of emotionally-relevant learning which is the MAAV test that is dependent on the amygdalo-hippocampal circuit functionality that is heavily affected by seizures of temporal lobe origin. In our study, NCSE has led to a severe deficit in the acquisition and retention of both auditory and contextual learning in rats that underwent two, more than one, episodes of NCSE pointing to the impact of

seizure burden. NCSE impaired rats' ability to associate auditory and contextual cues with the aversive stimulus (electrical shock) and develop an adaptive avoiding response that replaces innate freezing behaviors. Similar patterns of cognitive impairments have been reported in models of convulsive seizures, including those induced with KA, but not yet following NCSE (161, 162). The fact that our observed behavioral deficits were detected 1-7 days after the electroclinical offset of NCSE episodes points to early harmful mechanisms that are affecting the hippocampal integrity and may persist causing long-term consequences. Indeed, two episodes of NCSE resulted in a trend of decreased learning acquisition in the MAAV test one month post-NCSE. This pattern of learning may be comparable to that observed during early testing pointing to serious later life consequences of NCSE if proven statistically significant. In addition, later life cognitive and emotional deficits were observed in several behavioral panels one month post-NCSE echoing the neuropsychiatric comorbidities seen with epilepsy. In this extended experimental paradigm, two episodes of NCSE were shown to evoke impairments in contextual learning and visuospatial navigation indicating a lasting effect of NCSE on functions dependent on the amygdalo-hippocampal circuitry. Also, testing in the LD and OF tests pointed to decreased exploratory tendencies and anxiety-like behaviors after two episodes of NCSE in addition to decreased struggling in the FST which was indicative of possible depressive-like behaviors. Our findings are in line with clinically reported cases of chronic neuropsychiatric and cognitive deficits including memory impairments, anxiety, depression, and affective disturbances in patients with recurrent NCSE (158, 159, 163). Such comorbidities can be more debilitating than seizures themselves thus comes the importance of characterizing this model of NCSE.

Moreover, in this study, we provide evidence for potential neurobiological mechanisms underlying NCSE-induced behavioral deficits observed early in adolescence as well as later into adulthood. While it is commonly expected for prolonged seizures involving the medial temporal lobe structures to show extensive and gradually evolving hippocampal cell loss in a pattern resembling the one seen in human hippocampal sclerosis (164-167), there was no overt hippocampal cell loss detected in the early phase post-NCSE in this model as evidenced by NeuN immunostaining. Indeed, hippocampal NeuN-positive cell counts after one and two episodes of NCSE did not show a decrease in neuronal density. This might be explained in line with reports of absent hippocampal overt structural damage and neuronal cell loss following NCSE in animal models (168, 169). These findings are in line with existing clinical knowledge where post-mortem stereological studies report the absence of significant hippocampal neuronal loss despite the development of neurofunctional impairments in patients with a history of recurrent or prolonged seizures (170). However, neuronal damage can be subtle and not always detectable by NeuN staining, thus, it can be assumed that the observed hippocampal neuronal dysfunction is likely to be caused by other mechanisms such as axonal injury (171), which was beyond the scope of our investigations and is a subject of current work in our lab.

Another prominent morphological feature of TLE involving hippocampal sclerosis is reactive astrogliosis which is one of the hallmarks of seizure-induced hippocampal damage and a marker of epileptogenic brain tissues (172, 173). Here, we provide evidence for NCSE-induced hippocampal reactive astrogliosis evidenced by a marked increase in GFAP expression, a protein heavily expressed by astrocytes exhibiting hypertrophic changes in response to neuronal network hyperexcitability

(174). This increase in astrocytic density was detected in hippocampal CA2-CA3 and hilar regions early after two episodes of induced-NCSE implying an insult that can be attributed to homeostatic responses to hyperexcitability, acute neuroinflammation, or as a direct chemical effect of KA injections contributing to an epileptogenic brain network (174). Moreover, in the one month following NCSE, rats that underwent one and two episodes of NCSE had a tendency to exhibit electrographic spiking, which is a feature of hyperexcitable networks, that persisted for 4 weeks post-NCSE. However, the absence of spontaneous seizure recurrence was either due to slow spontaneous seizure emergence, which may occur as early as two weeks in convulsive SE models or after a relatively long latent period as seen in TPI models (175), or it might be possible that in the magnitude that we induced NCSE, it does not lead to spontaneous seizure recurrence, unlike CSE.

Furthermore, we also provide a novel finding of synaptic plasticity alteration in synaptophysin following two episodes of NCSE. Investigations of hippocampal synaptic plasticity showed a significant decrease in synaptophysin protein expression along the mossy fiber pathway from the dentate hilus into the CA2-CA3 subfields in brain sections of rats that experienced two NCSE episodes, but not one, correlating with more severe deficits in behavior. This protein is responsible for sustaining efficient synaptic neurotransmission and is suggested to play an integral role in hippocampal learning and memory by affecting dentate granule cells' ability to generate LTP (28, 32). Moreover, mutations in the synaptophysin encoding gene were reported in patients with intellectual disabilities accompanying epilepsy (33, 176). The herein observed alterations in GFAP and Syp protein expression profiles are suggestive of NCSE-induced maladaptive neuronal activation and network remodeling leading to possible

permanent functional disturbances and is heavily affected by seizure burden. Therefore, continued work in our lab is aiming at assessing the persistence of these molecular changes after one or two NCSE episodes in later life.

Our reported behavioral experimental outcomes combined with the neurobiological changes detected early after NCSE are clinically relevant and in line with emerging literature pointing to potential NCSE-induced lasting damage affecting the amygdalo-hippocampal circuitry in a way that might have detrimental consequences on the daily functioning of the affected individual. Indeed, a damage that is more pronounced after two episodes of NCSE hints to the harmful effect of seizure burden given that NCSE often recurs prior to coming to medical attention. If these results were confirmed, our findings call for an earlier diagnosis and a more aggressive treatment of NCSE.

Additionally, this study provides new insights into the mechanisms of synaptic facilitation with intriguing improvements in learning observed with certain subtypes of the MAAV test following one or two episodes of NCSE. First, an early enhancement in context-cued shock avoiding behaviors in the MAAV test was detected following one episode of NCSE. One hypothesis that may explain this improvement is synaptic tagging; by which a weak activation of synapses, for example by subconvulsive KA-injections, generates a synaptic tag that interacts with newly synthesized plasticity-related proteins (PRPs) (177). These proteins once captured at tagged sites during a proximate strong activation, as in the MAAV training, allow memory consolidation (177). This type of enhancement was described in several learning and memory models where it was shown that a weak learning task could result in long-lasting memory when followed by a stronger task within a critical time window (178-181). However, this

facilitation was not seen in rats that underwent a second NCSE episode possibly because of exacerbated maladaptive plasticity changes that altered the synaptic homeostatic balance in a way that led to dysfunction. Future studies will look into the role of PRPs especially since the changes in Syp expression levels did not parallel the behavioral changes observed in contextual learning. Another counter-intuitive performance enhancement in auditory learning acquisition was observed in the MAAV test one month post-NCSE in rats that underwent two episodes of NCSE. This enhancement can be attributed to a lesion-induced hippocampal behavioral facilitation effect (182). In the light of multiple studies that tested the hypothesis of hippocampal facilitation, it can be speculated that a lesion-induced impairments in rats' ability to exhibit unconditioned freezing in addition to disruptions in context-stimulus association have resulted in faster shuttling due to the lack of competition between the two responses (182-184). These phenomena require further exploration, making them the subject of ongoing investigations in our laboratory.

CHAPTER VI

LIMITATIONS AND FUTURE PERSPECTIVES

This work provides valid evidence on potential harmful consequences of NCSE on the involved brain regions in a manner that may affect individuals' healthy functioning. The study, however, has some limitations that can be traced back to time and financial restraints during the COVID-19 pandemic. Typically, studies of cognitive and emotional behaviors in animals require numbers not less than 15-20 animals per group in order to be sufficiently powered to establish statistical significance, hence, our long-term behavioral outcomes are still underpowered. Also, we here lack the possibility of intrahippocampal electrode recording, however, since the KA injections were directed to the hippocampus and seizure manifestations resemble those of hippocampal origin, the induced seizures can be strongly regarded as hippocampal NCSE. Ongoing experiments at our laboratory aim to increase the power of later life behavioral testing and further investigate the persistence of hippocampal molecular alterations.

The treatment of NCSE requires a multidisciplinary approach and needs to be tailored to the perceived urgency based on our knowledge of its harmful effects at different hierarchical levels of the brain. Further research is needed to confirm our long-term preliminary behavioral outcomes and support the assumption of long-lasting hippocampal structural and molecular changes following NCSE. Furthermore, since hippocampal neuronal loss was excluded here as a contributor to early observed behavioral deficits, alternatively, future histopathological investigations are needed to assess axonal integrity and functionality. Attention should be also given to NCSE-

induced circuit reorganization in various brain areas with a focus on activation patterns and synaptic plasticity changes that emerge during the latent phase following NCSE.

CHAPTER VII

CONCLUSIONS

In summary, NCSE has a negative impact on emotionally-relevant learning and memory accompanied by potentially permanent alterations in synaptic plasticity which probably have an effect on neuronal network homeostasis leading to more widespread damage. Indeed, our preliminary data on late NCSE effects point to a possible emergence of later life behavioral disturbances following recurrent episodes of NCSE. With this condition still being underappreciated in clinical practice, expanding knowledge of its detrimental consequences on cognitive and emotional well-being calls for a higher index of suspicion for its diagnosis and more urgent treatment approaches.

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