AMERICAN UNIVERSITY OF BEIRUT

A NOVEL PERI-ADOLESCENT MODEL OF HIPPOCAMPAL NON-CONVULSIVE STATUS EPILEPTICUS: EVIDENCE OF EARLY ALTERATIONS IN SYNAPTIC PLASTICITY AND COGNITIVE AND EMOTIONAL BEHAVIORS

by

RITA HAGOP ASDIKIAN

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"Alone we can do so little; together we can do so much" – Helen Keller

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AN ABSTRACT OF THE THESIS OF

Rita Hagop Asdikian for Master of Science
Major: Neuroscience

Title: A novel peri-adolescent model of hippocampal Non-Convulsive Status Epilepticus: Evidence of early alterations in synaptic plasticity and cognitive and emotional Behaviors

Background: Seizures consist of paroxysmal synchronous neuronal firing that may result in various signs and symptoms such as involuntary movements, abnormal sensations, and behavioral changes. While prolonged seizures accompanied by rhythmic clonic and/or tonic muscle stiffening (convulsions), termed convulsive status epilepticus, are known to be associated with mortality, brain damage, and chronic cognitive and psychiatric deficits, the effect of the non-convulsive type of status epilepticus (NCSE) on the brain remains largely unknown. Moreover, in current practice, the treatment approach to NCSE is less emergent compared to its convulsive counterpart, and its diagnosis is made relatively late, usually following multiple, often behaviorally subtle, episodes. This seemingly benign clinically non-dramatic condition has lately been the subject of few but alarming preclinical and clinical reports, suggesting NCSE-related brain damage, in addition to cognitive and emotional behavioral deficits. Confirming such potential detrimental consequences is highly clinically relevant. If NCSE has such major harmful sequealae, it should be diagnosed and treated more urgently, since in current clinical practice, it is considered less urgent than CSE. While much insight about CSE has been gained from animal models, expanding our knowledge about NCSE has been hindered by the lack of well-established animal models. Given that the temporal lobe is one of the most common sites of NCSE, here we propose to electroclinically validate a novel model of peri-adolescent hippocampal NCSE. Through this model, we aim at investigating potential cognitive, emotional-behavioral changes, as well as alterations in hippocampal structure and in synaptic plasticity markers, namely synaptophysin (Syp) and activity-related cytoskeletal (Arc) protein, following one or two episodes of NCSE, in this likely underdiagnosed condition that is usually brought to medical attention following multiple occurrences.

Methods: Postnatal day 43 (P43) rats received subconvulsive intrahippocampal kainic acid (KA) injections, in one dose (SKA group), or in two 24-hour apart doses (RKA group), under continuous EEG monitoring. Behaviors were monitored for two hours following KA

injections. Controls were sham manipulated following hippocampal cannula placement. RKA rats were subjected to a battery of behavioral tests 48 hours following the first KA injection. These tests were tailored to evaluate limbic functions, and included panels for exploratory tendencies, hyperactivity, and anxiety-like behaviors (light-dark box and open field tests), depressive-like behaviors (forced swim test) and hippocampal-dependent spatial navigation (Morris water maze). In addition, parallel groups of SKA and RKA rats were subjected to the modified active avoidance (MAAV) test. This test was developed in our laboratory to assess the recognition of auditory and contextual emotional cues, and the acquisition of adaptive electrical foot-shock avoiding behaviors. Following behavioral testing, structural hippocampal changes were analyzed via pyramidal neuronal counts, and immunohistochemistry for glial fibrillary acidic protein (GFAP). Protein levels of Arc and Syp were also histologically assessed.

Results: The subconvulsive dose of KA resulted in one (SKA) or two (RKA) episodes of electroclinical NCSE in 90% of the rats. Electrographically, each NCSE episode consisted of evolving rhythmic fast spikes and polyspikes between 20 and 130 minutes (average duration: 63.69 ±13.55 min). Behaviorally, NCSE was characterized by behavioral arrest, staring, oromotor automatisms, and salivation without progression to clonic activity. In the light-dark box test, compared to controls, the RKA group spent comparable durations in the lit chamber; however, they exhibited a decreased inter-chamber transition number. In the forced-swim-test, RKA rats exhibited decreased struggling and early learned despair compared to controls. In the MAAV test, two episodes of NCSE did not affect the rate of learning acquisition over the 6 training days, but resulted in a lower retention percentage in both tone-signaled and context-cued painful electrical-shock avoidance on the last test day. On the other hand, a single episode of hippocampal NCSE resulted in improved learning in both acquisition and retention subtests of the MAAV. Histological analysis revealed increased levels of Arc and Syp in rats that had undergone NCSE, more so in the SKA. There was no overt pyramidal neuronal loss in any of the groups.

Conclusions: Here we established the electro-clinical face validity of a novel reproducible rodent model that recapitulates the manifestations of temporal lobe NCSE seen in adolescents and adults. In line with emerging reports on potential harmful sequelae following NCSE, our preliminary work revealed depressive-like and anxiety-like behaviors, as well as deficits in emotional-relevant learning, following recurrent NCSE episodes. While a full behavioral testing panel following one NCSE episode has not been completed yet, there was an intriguing improvement in MAAV learning in this group of rats, potentially via hippocampal-related behavioral facilitation. In parallel to the behavioral changes following one or two episodes of NCSE, there was an NSCE-related maladaptive change in markers of synaptic plasticity. These results hint to potentially alarming cognitive and emotional behavioral derangements associated with NCSE that, if confirmed, call for a more urgent diagnostic consideration and treatment to this currently underdiagnosed condition. Ongoing work aims at confirming the potentially harmful effects of recurrent NCSE in the herein described novel model, and at investigating the mechanistic underpinnings of the observed counter-intuitive behavioral facilitation following a single NCSE episode.

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Arc	Activity-regulated cytoskeletal-associated protein
CA	Cornu Ammonis
CREB	cAMP response element binding protein
CSE	Convulsive Status Epilepticus
CTR	Control
DG	Dentate Gyrus
EEG	Electroencephalography
EPSP	Excitatory Postsynaptic Potential
FST	Forced Swim test
GABA	Gamma-Aminobutyric acid
GFAP	Glial Fibrillary Acidic Protein
IPSP	Inhibitory Postsynaptic Potential
KA	Kainic Acid
KAR	Kainate receptor
LDT	Light-Dark Box Test
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
RKA	Repeated Kainic Acid
SE	Status Epilepticus

SKA	Single Kainic Acid
Syp	Synaptophysin
TLE	Temporal Lobe Epilepsy

CHAPTER I

INTRODUCTION

The central nervous system is one of the least understood structures of the human body, and its exploration is one of the relatively most recent fields in biological sciences. The main functions of the central nervous system in general, and the brain in particular, are to aid in maintaining internal bodily homeostasis, and in regulating adaptability of the organism to the external environment (1,2). In addition to controlling and regulating essential bodily functions, the brain processes environmental cues and information, and tailors responses accordingly. The fulfillment of these functions and the maintenance of structural and functional integrity are largely dependent on a delicate electrophysiological balance. The disruption of this balance, due to genetic or acquired factors, leads to various pathological conditions, the prototype of which is seizure emergence. A seizure consists of excessive synchronous neural firings that may behaviorally manifest with subjective sensations or involuntary muscle activity, which in many cases culminate into rhythmic-clonic or tonic muscle stiffening, known as convulsions. While transient seizures may occur as a manifestation of an acute provocation (trauma, infection, or fever in toddlers…); some individuals have an acquired or innate epileptogenic network, and may suffer from chronic recurrent seizures, a phenomenon termed ‘’Epilepsy’’. Epileptic disorders burden the daily functioning of the affected individual. Indeed, it is often accompanied by cognitive and psychiatric comorbidities, and may be aggravated by prolonged epileptic episodes, called Status Epilepticus (SE), which is a life-threatening medical emergency. Aside from possible serious physical injuries and an overall poor quality of life, epilepsy is

associated with a disruption in brain functions, often in the brain region from which it originates. This is particularly true for seizures originating from the temporal lobe that houses the amygdalohippocampal circuitry; a structure subserving emotionally-relevant learning and memory, and highly conserved within species through evolution (3,4). This circuitry has a key role in explicit memory formation, language, and emotional regulation, orchestrating many cognitive responses and emotional adaptability to new environments and learning tasks that require pattern recognition and relational processing. In rodents, its function can be more specifically described as processing survival-driven relational patterns of environmental cues as in context-cued conditioning tasks or cues-dependent visuospatial navigation (5-7). The disruption of the normal function of this circuitry may be associated with cognitive and emotional behavioral disturbances as well as the emergence of temporal lobe epilepsy (TLE); the most common cause of treatment-resistant seizures in adolescents (8,9). In addition to pharmacoresistance, TLE is associated with cognitive and psychiatric comorbidities reaching a high 40% risk of anxiety and depression in TLE patients (10). Aside from convulsive episodes, seizures may come upon in many cases without convulsions. In TLE, the non-convulsive seizures take many forms like paroxysmal language difficulties, behavioral arrest, fluctuating consciousness, and automatisms. Since these non-convulsive seizures are often difficult to detect by observation and thus may be overlooked, it is believed that they are underdiagnosed or at least diagnostically considered late after multiple recurrences, resulting in late anti-seizure treatment initiation. While a number of alarming clinical and preclinical reports suggest that these non-convulsive seizures, when prolonged, can have harmful effects on brain structure and on behaviors (11,12), their potential detrimental effects remain unconfirmed. Should prolonged non-

convulsive seizures - termed Non-Convulsive Status Epilepticus (NCSE) - have harmful consequences, they need to be diagnosed and treated more urgently than what is being done in current clinical practice.

Diagnosis, treatment, and prognosis are the essential building blocks of applied medicine, insight in which is often gained from animal models, especially in specific conditions like SE, the main subject in our research laboratory. Given the remarkable anatomical and physiological resemblance between humans and other mammals, researchers have long relied on animal models to investigate the underlying mechanisms of disease processes, and to assess novel therapies in order to apply and extrapolate their findings to humans. While much insight can be gained from such correlations between humans and animals in many diseases, others are still under-explored experimentally, as is the case with NCSE. Given the lack of well-established animal models, our focus in this study is to develop a novel peri-adolescent rodent model of NCSE of temporal lobe origin. We aimed at analyzing the effects of hippocampal NCSE on synaptic plasticity, and on functions mostly associated with the amygdalohippocampal circuitry, namely emotionally-relevant learning and memory, spatial navigation and emotional behavioral adaptability to new environments by assessing depressive and anxiety-like behaviors.

Below is a review of the epilepsies, with a focus on temporal lobe seizures, and a description of the amygdalohippocampal circuitry role in cognitive and emotional behaviors. We will then review NCSE, a seemingly benign condition, in the light of accumulating reports on its harmful effects, with a special attention to its potential consequences on amygdalohippocampal functions, a field not yet thoroughly explored, and

serving as the basis of our research questions. We will then review experimental animal seizure models, zooming in on the platform we employed to launch our new model, and on the nature of the behavioral tests used to asses our research questions.

A. Seizures and Epilepsy: a disruption in electrophysiological homeostasis

As mentioned above, the brain requires the maintenance of electrophysiological balance within a specific range in order to maintain its functions and preserve the integrity of its structures (1). The disruption of electrophysiological homeostasis leads to aberrant neural signaling causing a variety of disorders, notably seizure emergence. A seizure consists of paroxysmal excessive neuronal firing in the brain; a condition in which neurons become synchronously active due to temporary disruption of neuronal electrical homeostasis. Recurrent seizures result in a chronic condition termed ‘’Epilepsy’’ often debilitating due to the risk of physical injury, potential neuronal damage, and cognitive psychiatric comorbidities. In terms of prevalence, epilepsy is one of the most common brain disorders, affecting approximately 65 million people of all ages (13), with a high incidence in childhood, reaching up to 1% of children worldwide (14). Indeed, epilepsy is one of the leading contributors to mental disability and early mortality, ranking fifth among mental health, neurological, and substance-use disorders in low- and middle-income countries and the seventh worldwide (15). Moreover, epilepsy has a global disease burden equal to lung cancer in men and breast cancer in women (16).

There are many innate and acquired causes for epileptic conditions (Figure 1), but they do have commonalities, as discussed below, in the disruption of electrophysiological

homeostasis, and the accompanying electroencephalography (EEG) changes and clinical manifestations (17). Mechanistically, irrespective of the underlying etiology, there is probably a common end pathway that results in hyperexcitability, and a dysregulation in the normal balance between the main excitatory and inhibitory mechanisms in the brain (17). The main channels involved in maintaining this delicate electrophysiological homeostasis are the inhibitory channels $GABA_A$ (gamma-amino-butyric acid) ionotropic receptors, NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, and kainate receptors (KAR) (17). The various epileptic aberrant mechanisms eventually exert a net shift in the electrophysiological homeostasis towards hyperexcitability. In the clinical arena, this hallmark feature of epilepsy is assessed via EEG. Normal neuronal functioning is the result of continuous interactions between neurons. The EEG measures excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) (18,19). Disturbances in EPSPs and IPSPs results in paroxysmal neuronal firing that is reflected on EEG with abnormal electrical waves in patients with epilepsy. Electrophysiological disruption between seizures consist of spikes and sharp waves (morphologically pointed waves that last less than 80 msec or more respectively). During seizures, on the other hand, the EEG displays rhythmic evolving activity, usually with spikes and sharp waves components. When electrographic seizure patterns are accompanied by behavioral paroxysms, they are termed electroclinical seizures. These paroxysms may consist of subjective sensations (auras), smells or stomach discomfort, behavioral arrest, and in their most dramatic forms, seizures may manifest with motoric changes characterized by involuntary muscle activity (20,21). While muscle activity can be as subtle as myoclonic jerks (behaviorally startle-like and less than 200

msec in duration), they could also take the form of easily detectable behaviorally prominent rhythmic-clonic, or tonic muscle stiffening termed convulsions (22). Seizures that are not accompanied by convulsions (the non-convulsive types), take the form of various often subjective, behavioral paroxysms, such as behavioral arrest, language difficulties, automatisms, etc. Therefore, these are often difficult to detect by an observer, and only EEG can provide the clinician with a definitive diagnosis.

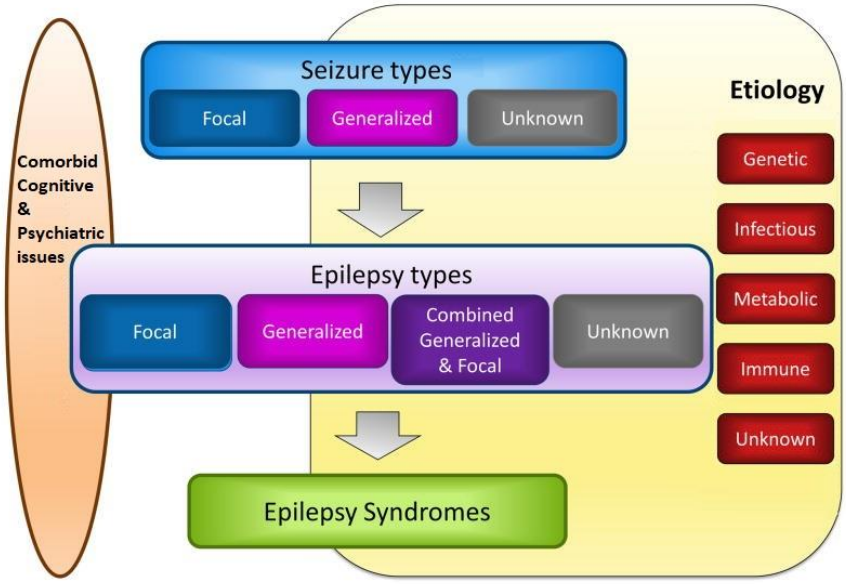

Figure 1. Illustration of the various etiologies of the epilepsies, and the various factors and modifiers that help in classifying them. Modified from ILAE classification of the epilepsies 2017 (23).

In addition to etiological classification of the epilepsies, seizures can also be electroclinically classified depending on the accompanying clinical signs and symptoms, and on whether they start focally or simultaneously in both cerebral hemispheres (Figures 2

and 3). If the seizure originates in on one hemisphere or in one lobe, the seizure is classified as focal. This is in contrast with generalized seizures that involve both hemispheres at onset (24). Nonetheless, focal seizures can also spread to involve the contralateral hemisphere (secondary generalization). Since focal seizures start in a specific abnormal electrical network focus in the brain, they often display signs specific to the function of that specific area. For example, seizures arising from an electrical focus in the frontal lobe can result in unusual head and eye movements, abnormal posturing, or at times, paroxysms of unexplained behaviors and uncharacteristic personality changes. On the other hand, temporal lobe seizures may manifest with auras of visual or auditory hallucinations, more commonly epigastric sensations and déjà-vu illusions, fluctuation of alertness or behaviors, in addition to more prominent muscle activity in case the electrical activity spreads to motor areas (20,21). In addition, seizures are also believed to disrupt the function of the network from which they originate; a negative effect mostly studied in seizures that are prolonged in the form of CSE (25-27). However, the effect of prolonged non-convulsive seizures on brain network functions remains elusive despite some alarming reports on NCSE-related brain dysfunction and damage. One of the most common sites of NCSE in adolescents and adults is the temporal lobe. Whether hippocampal NCSE results in interictal dysfunction in this structure heavily involved in cognitive and emotional regulation remains unconfirmed. However, a potential harmful effect of NCSE is suggested by the multitude of comorbidities that occur in patients with temporal lobe seizures. In what follows, is a discussion of temporal lobe epilepsy and the accompanying dysfunction in the amygdalohippocampal circuitry, shedding the light on the importance of this circuitry in cognitive and emotional behavioral regulation and adaptability.

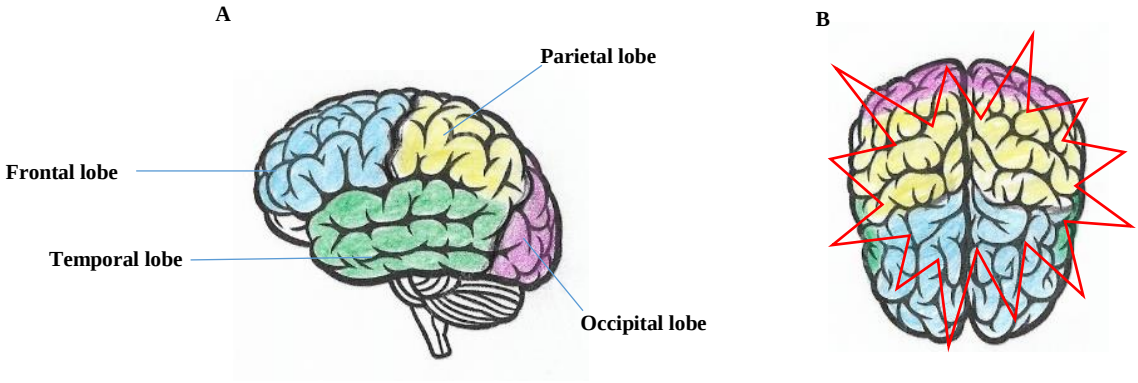

Figure 2. Schematic illustration of focal and generalized modes of seizure onset. Panel A. Focal seizures arise in one hemisphere or in one lobe (frontal, temporal, parietal, or occipital), and may or may not spread to the contralateral hemisphere. **Panel B**. Generalized seizures are characterized by an abnormal neuronal firing that simultaneously recruits networks in both hemispheres at seizure onset. If seizure onset is focal (Panel A) and then spreads to the contralateral hemisphere (Panel B), it is classified as a secondary generalized seizure. However, when both hemispheres are affected at onset, the phenomenon is termed a primary generalized seizure.

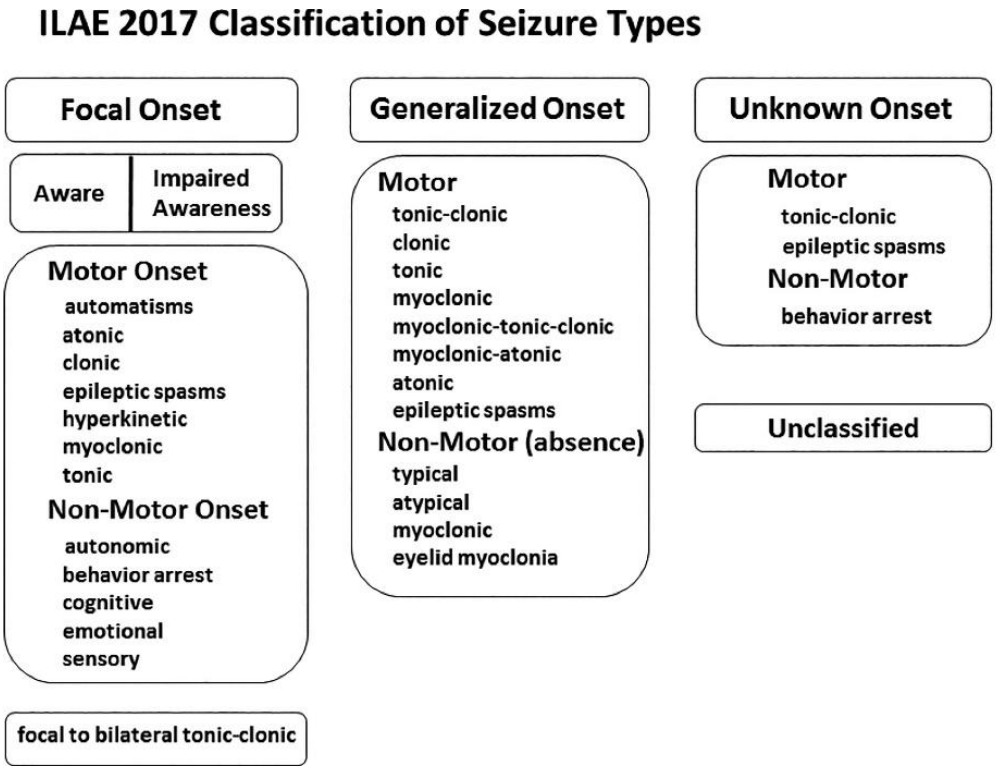

Figure 3. Seizures classifications based on electrophysiological mode of onset and on clinical manifestations, modified from the 2017 ILAE classification of the epilepsies (23).

B. Temporal Lobe Epilepsy: a prevalent type of treatment-resistant focal epilepsy

Temporal lobe epilepsy often recruits the amygdalohippocampal circuitry, with various etiologies such as hippocampal sclerosis, cerebral malformations, infections, hypoxia, and trauma. In most cases of TLE in adolescents and adults, seizures are associated with hippocampal sclerosis (Figure 4) while in children younger than 5 years old, pathologies are usually related to cerebral malformations, though at times accompanied by anatomical hippocampal changes (9,28-30). TLE clinically manifests with recurrent seizures that are often treatment resistant, notably in adolescence, and especially when associated with hippocampal sclerosis, where up to 70% of patients may not respond to anti-seizure medications (31-33). During a seizure, affected patients may experience auras of epigastric sensations, feelings of fear (10), and paroxysmal emotional changes (34). Auras can be accompanied or followed by unusual mouth movements, motionless staring, and possibly partial tonic movements, that may culminate in full-body convulsions (20,21).

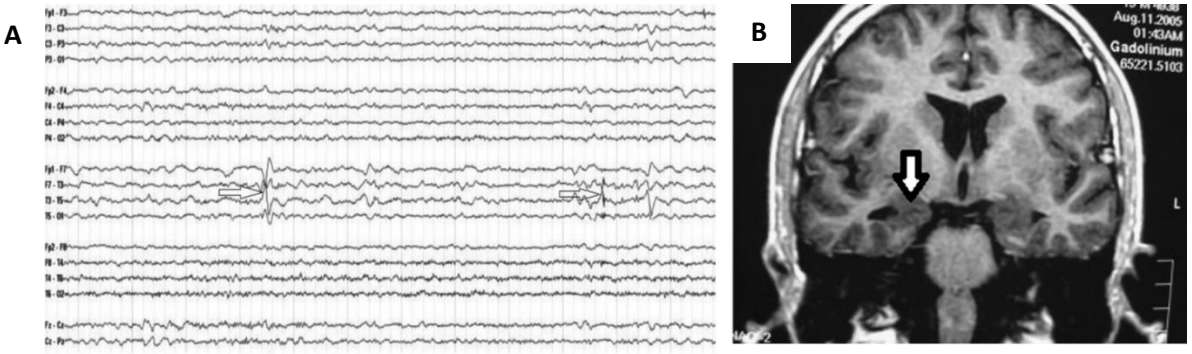

Figure 4. Electrographic and imaging changes in a patient with TLE (copyright obtained). **Panel A.** Interictal epileptiform features in the left temporal area (35). **Panel B.** MRI representation of right hippocampal atrophy (white arrow) in a child with temporal lobe epilepsy (36).

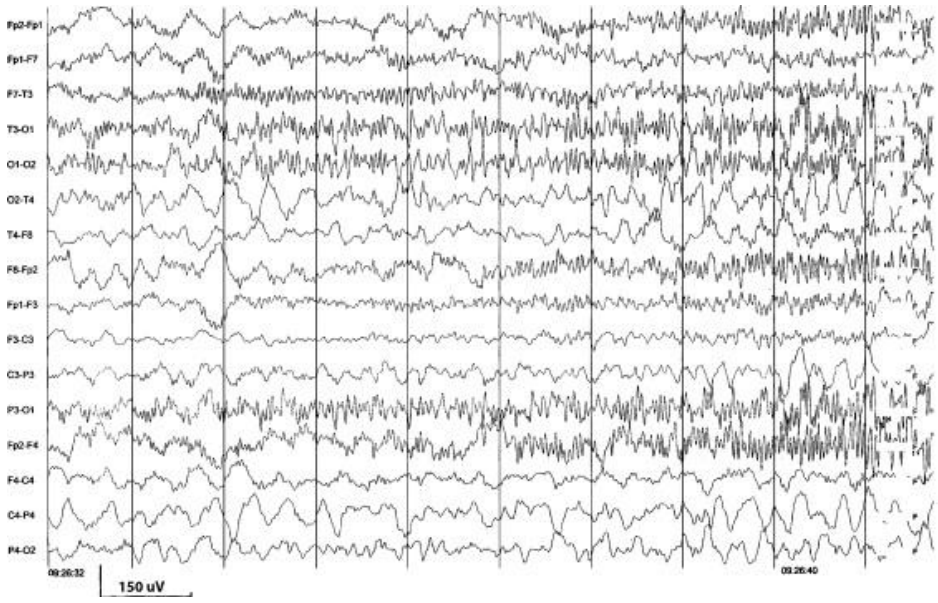

Figure 5. The electrographic pattern of a seizure arising from the left temporal lobe with fast rhythmic spikes and ubsequent spread to the right frontal area (36) (copyright obtained).

1. TLE comorbidities

In addition to pharmacoresistant seizures, TLE patients suffer from cognitive dysfunction, as well as psychiatric comorbidities such as anxiety, and depression, that commonly occur in the adolescent age group (37-41). Indeed, TLE is notorious for leading to derangements in learning and memory and emotional regulation, in addition to cognitive disabilities most commonly in the form of reading disabilities that occur in up to 30% of children with TLE (42). Other cognitive disabilities include poor performance in arithmetic, spelling and word recognition (33,42,43). Parents of those children also report problems in attention and coordination, in addition to various degrees of hyperactivity (44). Moreover, in visuomotor coordination tasks (eye-hand coordination), children perform worse than their normal counterparts (43), even in the presence of a normal IQ. Moreover, it was shown that the longer the duration of TLE, the greater the impact on amygdalohippocampal

functioning, specifically cognitive performance (34,45,46) with an alarming increase in suicidal attempts (50,51). In addition to cognitive disturbances, TLE is also associated with depression and anxiety (10,47-49). Interictal anxiety and panic attacks occur frequently in patients with partial seizures in general, and even more so in seizures arising from the temporal area. In addition TLE patients present with a high 40% risk of depression (10).

The cognitive and psychiatric comorbidities of TLE, and epilepsy in general, are multifactorial in nature (52-57). Contributing factors include the presence of a lesion and its type, the number and types of anti-seizure medications, seizure frequency, and social and genetic factors. This is particularly true in TLE that houses many key functions in emotional regulation, and cognitive adaptability, especially that it is often treatment-resistant leading to a high seizure burden, and to excessive drug-related adverse effects. The unpredictable nature of seizures often lead to a sense of insecurity and lowered self-worth due to fear of having seizures in public, in the face of a stigmatizing social attitude (34,58). The fact that TLE is accompanied by a multitude of cognitive and psychiatric comorbidities can be explained to a large extent by the well-known key cognitive and emotional regulatory functions of the amygdalohippocampal circuitry discussed below.

C. Amygdalohippocampal circuitry: a major contributor to cognitive and emotional regulation

1. The Hippocampus

The hippocampus is a bilateral structure underneath the cortical surface, located in the medial temporal lobe of the brain. This curved structure that looks like a seahorse

(hippocampus in Greek), has a key function the formation of declarative memory and in spatial navigation (7). In neurodegenerative diseases such as Alzheimer's disease, the hippocampus is one of the first regions of the brain to be affected, leading to declarative memory loss and disorientation associated with the condition. The hippocampal formation comprises the *Cornu Ammonis* (CA) regions (hippocampus proper), that contain pyramidal neurons, and the dentate gyrus (DG), which consists of granule cells (59-61). The anatomical arrangement of the CA and DG in a curled structure makes these regions qualified targets for both *in vivo* and *in vitro* electrophysiological studies (59). The CA region is further divided into three areas containing pyramidal neurons: CA1, CA2 and CA3; and a final CA4 layer containing polymorphic cells and located at the end portion within the blades of the DG. The CA4 layer is considered a part of the dentate gyrus and often referred to as the hilar region. The main connections of the CA4 region are from and to the dentate gyrus via mossy fibers (62).

Historically, CA1 was the first region identified in the hippocampal output circuitry. Pyramidal neurons within this region are characterized by significant synaptic plasticity, and are organized and associated with one another along the longitudinal axis of the hippocampus, providing a reliable signal transfer among those cells (60). The CA1 region gives rise to a major output pathway from the hippocampus towards layer V of the entorhinal cortex (Figure 6) and to the subiculum. CA2 is a small hippocampal region located next to CA1. The major input into this region comes from layer II of the entorhinal cortex via the perforant path. Similarly to CA3 and unlike CA1, pyramidal cells of this region are less densely packed, and have fewer dendritic ramifications (63). The CA3

region receives input from the entorhinal cortex via the perforant path, and from the mossy fibers of the granule cells in the dentate gyrus. Output pathways of this region go back to the dentate gyrus hilus, and mostly to regions CA2 and CA1 via the Schaffer collaterals (Figure 6). CA1, along with CA3, has been shown to contribute in the synchronous bursting of electrical discharges associated with interictal epileptiform activity (59).

In line with its role in declarative or verbal memory formation described in humans, a growing body of evidence from animal studies emphasizes the role of the hippocampus in pattern separation especially in anxiety-provoking situations. In other words, the hippocampus has shown a capacity in making spatial and visual object associations, thus relating environmental cues allowing visuospatial navigation in tests such as the water maze (7) and contextual learning in conditioning testing paradigms (59,64-66). As discussed later below, these functions are often disturbed in seizure animal models.

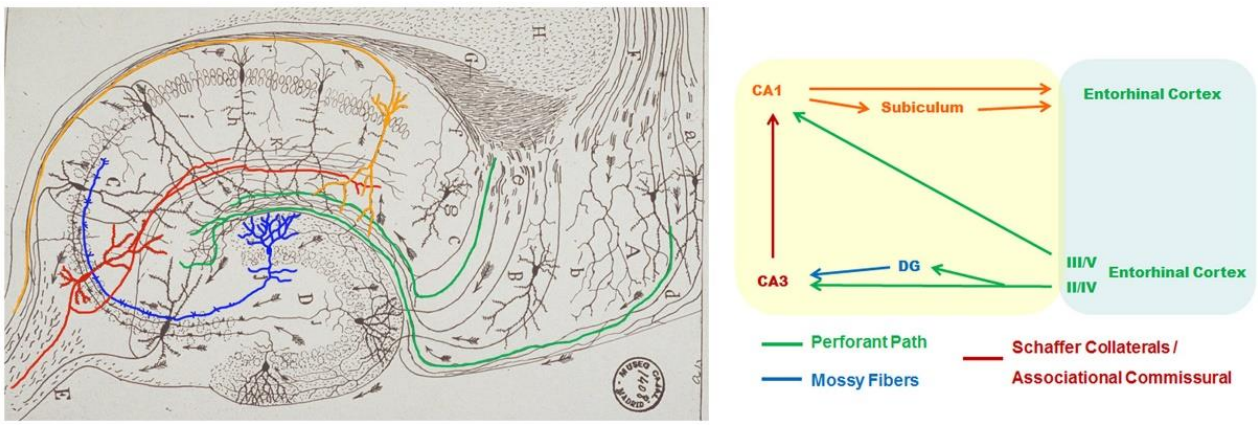

Figure 6. Illustration of the hippocampal neuronal circuitry. Pyramidal neurons in the hippocampus are densely packed in one layer which is divided into several regions with CA1 (in orange) and CA3 (in red) being the major ones. Pyramidal neurons of the CA3 region receive excitatory stimulations from both the entorhinal cortex and dentate gyrus via the perforant path (in green) and mossy fibers (in blue) respectively. Axons of the CA3 cells or Schaffer collaterals stimulate dendrites of the CA1 region forming the stratum radiatum. CA1 axons send excitatory impulses back towards the entorhinal cortex (67).

2. The amygdala and survival-driven emotional learning

In humans, the amygdala is an almond-shaped structure located bilaterally in the medial temporal lobe of the brain. It is one of the primary structures involved in emotional processing, specifically fear, and in tailoring responses to emotional threats (68,69). In animals, this structure has been the main subject of fear and survival-related conditioning experiments (70). Fear emotions in survival-relevant situations accelerate the learning process. They are automatic brain responses that can be triggered by challenging tasks, and exposure to new environments. Conserved throughout the evolution of species, emotional experiences especially those with a survival value are characterized by a high capacity of mediating cognitive processes, perception, learning and memory (71). Most emotions are mediated by the amygdala with a humble participation of the cerebral cortex (72). In fact, behavioral and physiological analyses following amygdalar lesions show blunted emotional reactions to normally threatening stimuli, implying that the amygdala is an essential component in the emotional processing of the conditioned fear pathway. Indeed, the amygdala, specifically its basolateral nucleus, contains neurons that make an association between a conditioned stimulus, such as a tone or a visual cue, and certain aversive unconditioned cues (pain foot shock) that are associated with innate responses, in a process referred to as ‘’fear conditioning’’.

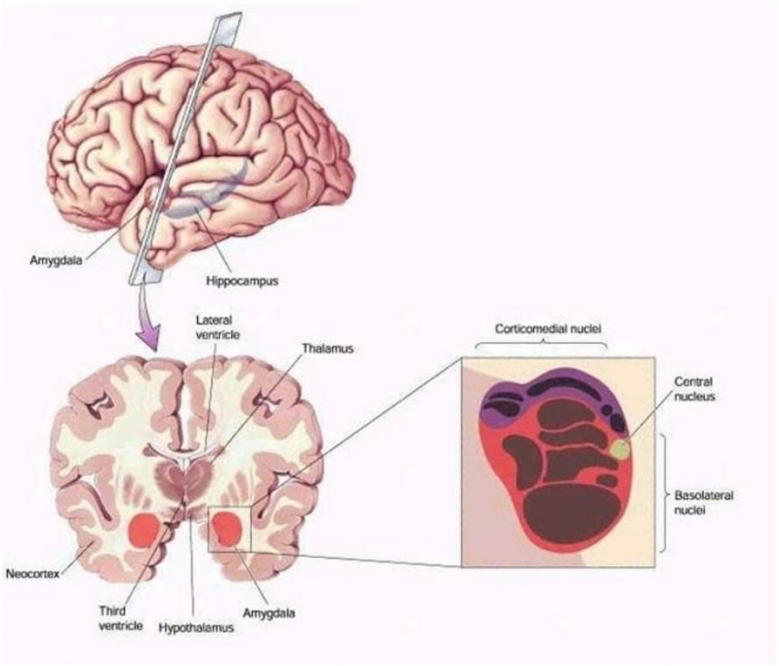

Figure 7. Illustration of the anatomy of the amygdala. In a coronal human brain section, the amygdala or amygdaloid complex appears to be a complex mass of gray matter formed by three distinct groups of subnuclei. The corticomedial nuclei connect with the olfactory bulb and cortex, the central nuclei however, connect with the brainstem and hypothalamus whereas the basolateral nuclei are characterized by rich connections with the cerebral cortex mainly the prefrontal area (67).

D. Molecular and structural underpinnings of learning and memory

The brain receives information from its surrounding environment and consequently customizes responses accordingly. These behavioral responses can be learned or innate in nature. The acquisition of learned responses relies on intricate and so far not completely understood mechanisms that encode specific information via changes in synaptic transmission and neuronal excitability in certain neuronal networks. Some of the molecular underpinnings of changes in neuronal excitability were shown to be triggered by the transcription factor cyclic AMP-responsive element-binding protein (CREB), hinting to the probability that a given neuron will be involved in storing a specific information

(73,74). At the network level, CREB also contributes in learning acquisition (73,75) and allocating information to specific areas in the hippocampus in a process referred to as neuronal network allocation. Transduction of CA1 neurons with virus-encoded CREB leads to an enhancement in learning and memory in contextual conditioning (76) or in the Morris Water Maze (77), two panels related to hippocampal-dependent learning. Interestingly, post-training transduction attempts with virus-encoded CREB fails to alter memory performance (77), implying that the enhancement observed was cell-specific and potentially due to the "state of readiness" of neuronal networks with increased CREB. Similarly to the amygdala, it has been shown that memory allocation in the hippocampus is also modulated by increased excitability due to activated CREB, with reduced after hyperpolarization currents in hippocampal CA1 pyramidal neurons, hence reduced thresholds for long-term potentiation (LTP) (78). The importance of these findings lie in the fact that LTP is considered as one of the electrophysiological surrogates of learning and memory (70,71), and in simpler terms it refers to a repeated firing-related enhanced synaptic transmission between two given neurons. Following intense stimulation of the presynaptic neuron, the amplitude of the post-synaptic neuron's response increases, leading to sufficient depolarization, and further activation of post-synaptic signaling cascades (79). Therefore, lower LTP thresholds could potentially imply neural activation with less stimulation intensity. While a multitude of electrophysiological and molecular surrogates of synaptic plasticity and learning acquisition have been studied, we will focus in the following subsections on two protein markers that have been frequently described both in seizure models and in learning paradigms.

1. The Activity-Regulated Cytoskeletal-Associated Protein (Arc/Arg3.1)

Multiple molecular cascades have been described in learning, memory and conditioning paradigms. The cAMP-ERK-CREB signaling cascade is one of the most studied pathways in fear conditioning, including its downstream effector, the activity-regulated cytoskeletal-associated protein (Arc). Arc is an immediate early gene expressed in glutamatergic neurons in response to neural activities involved in learning (80-83). Being tightly linked to neuronal activity, Arc expression has been extensively used as a tool to study both functional and dysfunctional neurons. Moreover, levels of Arc induction vary with different brain regions and cell types (84,85), mirroring their activity levels and hence their rates of contribution in a given behavioral task. Arc protein trafficking and localization is also highly selective and well regulated. It can be found in dendrites (86,87), in the nuclei of neurons (88,89) and in post-synaptic densities of recently potentiated synapses (86,90,91), where it can function in interacting with structural proteins, and with proteins critical to synaptic plasticity (92,93). Molecularly, Arc participates in the regulation of AMPA-type glutamate receptors (AMPARs) endocytosis (94,95), and of spine size and type (96), which likely hint to its important role in learning, memory and synaptic plasticity. At the electrophysiological level, Arc expression is induced by LTP (98), long-term depression (LTD) (99), and by seizures (98,100). Behaviorally, the levels of Arc expression have been shown to correlate with the rate of learning acquisition in various paradigms. Indeed, animals with high levels of Arc mRNA expression in the striatum or hippocampus were shown to have faster learning in a reversal motor-response task (75), or in spatial learning tasks (82,97).

2. Synaptophysin

Synaptophysin I (Syp) is a membrane protein expressed on synaptic vesicles, the most abundant subcellular structures in the mammalian nervous system (101,102); hence, Syp is ubiquitously expressed throughout the brain. Molecular studies have identified potential functions of Syp in exocytosis, synapse formation, and endocytosis of synaptic vesicles (103,104). From a behavioral aspect, experimental studies on Syp knockout mice did not result in mortality (98), but lead to dramatic behavioral changes consistent with cognitive disability and learning deficits (105,106). These findings point to a key role of this synaptic component in neuronal communications in circuits of learning and memory, even though the likely multiple intricacies of its exact role remain to be further molecularly dissected.

E. Status Epilepticus

Prolonged seizures are referred to as status epilepticus (SE), a condition in which the subject experiences a prolonged seizure or recurrent seizures for over 30 minutes with no recovery of consciousness in between the epileptic episodes; these types of seizures are believed to disrupt amygdalohippocampal functions and to be likely implicated in the cognitive and emotional deficits that arise in patients with TLE (107-111). The definition of SE has been subjected to multiple modifications that paralleled our improved understanding of its underlying molecular and pathological mechanisms; however, it is well accepted in research to consider timeframes-based definitions (30 minutes) in order to study and compare the effects of seizures on the brain in clinical and preclinical studies. Convulsive

status epilepticus (CSE) is the most common pediatric neurological nonsurgical emergency with a prevalence that ranges between 6 and 20 per 100,000 yearly, with an overall etiology-dependent mortality that ranges between 3 and 20 % (112,113). Besides the acute CSE-related mortality, it is associated with high morbidity (114). Identification and treatment of CSE is a medical emergency since prolonged CSE can lead to various physiological derangements, but most importantly to permanent brain damage, particularly after continuous convulsive seizures for more than 30 minutes based on pathological studies in baboons (115,116). In addition to well established brain damage and mortality is it well accepted that CSE is associated with cognitive and psychiatric comorbidities, and the longer the duration, the more harmful the sequelae (25-27). In contrast to CSE, NCSE is characterized by electrographic seizures without tonic-clonic activity, motorically only manifesting with subtle motor signs like twitching and blinking, but mainly paroxysmal behavioral changes such as altered mental status, behavioral arrest, and automatisms (117-119). Moreover, while CSE has been relatively well characterized in established animal models, and epidemiologically studied in humans, the non-dramatic form of SE, NCSE remains largely understudied. In the following section, this poorly recognized and studied seemingly benign condition will be reviewed in the light of alarming though unconfirmed reports on its harmful effects on the brain.

F. Non-Convulsive Status Epilepticus: an underdiagnosed potentially harmful condition

NCSE is likely one of the most underdiagnosed conditions. Similarly to CSE, NCSE can be focal or generalized. Indeed, NCSE in the pediatric population occurs in the setting of generalized epilepsies or can be focal in origin, most commonly in adolescents and adults with TLE. Generalized NCSE occurs in the context of typical or atypical absence seizures in children with childhood absence seizure syndrome or the more severe Lennox-Gastaut syndrome respectively (12,120). Concerning NCSE of focal origin (complex partial seizures), it can follow partially treated CSE, it can be the manifestation of a preexisting epilepsy, or it can emerge as a de novo manifestation in the form of the first lifetime seizure. In current clinical practice, CSE is considered an emergency and has to be aborted within minutes (even if anesthetic agents and mechanical ventilation are required), while NCSE is approached non-urgently with drug titrations over hours and sometimes days (121). It is noteworthy that NCSE in the context of partially treated CSE has gained a heightened diagnostic awareness. On the other hand, de novo NCSE or NCSE in patients with a history of epilepsy are still not urgently diagnostically considered and treated in current practice. They are also widely underdiagnosed and non-convulsive episodes likely recur over days prior to coming to medical attention and therapeutic control (121). In addition to this lack of prompt diagnostic consideration, the absence of well-established diagnostic criteria of this condition, and the heterogeneities in epidemiologic studies, may explain the low reported incidence in the literature. In fact, the non-convulsive subtype of SE was not included in the International Classification of Disease-10 (ICD10) up until 1996. Nonetheless, based on recently reported cases, the prevalence of NCSE can be

assumed to be around 3-15 per 100,000 population yearly, compared to a CSE prevalence of 6-20 per 100,000 yearly only in the pediatric population, and in general up to 50 per 100,000 yearly in industrialized countries (122-127). Despite reports about an associated with high mortality rate (128-130), the potential harmful cognitive and psychiatric o this under-recognized condition remain largely unknown as discussed below.

NCSE is characterized by protean focus-specific presentations that are often difficult to clinically recognize, such as confusion, behavioral arrest, automatisms and paroxysms of emotional changes, in addition to subtle, if any, motor signs namely myoclonic twitching and blinking (117-119). Therefore, most cases of NCSE can only be identified and confirmed by electrographically detecting the seizures on EEG during the episodes. This at times requires prolonged continuous EEG monitoring over hours to days in order to electrographically capture and confirm the nature of events suspected to be epileptic in origin. Consequently, this condition comes to medical attention relatively late, and after multiple occurrences. While underdiagnosed, undertreated, and lacking well-defined therapeutic approaches, few but alarming accumulating reports point to concerning evidence on potential detrimental structural and behavioral NCSE-related changes. Indeed, some studies reported NCSE-related brain damage. Structural alterations in the temporal cortex and the thalamus have been described on magnetic resonance imaging (MRI) during ongoing NCSE in the form of DWI (diffusion-weighed imaging) hyperintensities (131). Also, post-NCSE increased MRI signal in the temporal lobes has been reported in one patient with a known history of complex partial seizures of temporal origin (132). In addition, increases in neuron-specific enolase, a markers of acute brain injury, has been

described in NCSE patients (133,134). Such potential detrimental NCSE-related consequences are important to confirm in order to provide substantial scientific proof-based support to diagnostic and therapeutic approaches to NCSE, potentially stressing the importance of a more urgent diagnosis and treatment of this condition.

Behaviorally, NCSE symptoms vary depending on the network of origin. Although it has been widely shown that NCSE commonly originates in the temporal lobe (121,135-138), it can also arise from extratemporal areas, such as from the frontal lobes (121). An electrical focus in the frontal lobe can result in paroxysmal uncharacteristic personality changes and unexplained social conduct. On the other hand, NCSE of the temporal lobe presents with acute paroxysmal changes related to amygdalohippocampal circuitry dysfunction, such as problems with memory in addition to emotional disturbances. Patients may experience forgetfulness, difficulty finding words, periods of ‘’zoning out’’ or being mentally absent (12,120,139-141). Since NCSE has protean presentations, the aforementioned manifestations are often attributed to more common and stereotyped conditions such as dyslexia and autistic features (142). These clinical commonalities may explain why in current clinical practice, NCSE is not only rarely included in differential diagnoses of a given behavioral or cognitive paroxysm, but also considered late in the clinical course. Moreover, there is currently no specific NCSE-tailored approach, and a general lack of well-established treatment protocols.

In addition to the lack of substantial clinical data on NCSE and its effects on the brain, expanding our knowledge on this condition in the pre-clinical arena has been also hindered by the lack of well-established animal models. Unlike CSE that has been well

characterized in rodent models in translational epilepsy laboratories including ours (8,143,144), there is a desperate need for electroclinically valid NCSE models. There have been only few attempts to establish such models in relatively recent emerging reports. Since NCSE commonly emerges from the temporal lobe, some investigators aimed at developing animal models that mimic the electroclinical manifestation of hippocampal NCSE. In rodents, the mammals most studied in epilepsy, electrical stimulation has been attempted in one study, as well as systemic pilocarpine in another, with some success in electroclinically reproducing NCSE (145,146). In another study, a targeted hippocampal delivery has been attempted in pigs (147), but not yet in rats. Not only do rats provide a reliable platform to study seizures, but they also offer the advantage of ease of manipulation, and the possibility of comparing study results to an existing research database, and studying surrogates of human cognitive and psychiatric epilepsy comorbidities in well-established behavioral testing paradigms. Below is a review of existing rodent seizure models that helped us in launching our NCSE model.

G. Preclinical models of hippocampal SE

The cognitive and emotional behavioral disturbances associated with temporal lobe seizures in children represent a heterogeneous group in terms of contributing factors that include seizure type, frequency, as well as the age of onset and duration of the seizure disorder (55,57). This is in addition to the potentially harmful contribution of interictal EEG activity (52,55,56,148), and anti-seizure medication burden (53,54,149-153). The relationship between these variables, intellectual ability, psychiatric comorbidities and

academic performance has received considerable attention from researchers. As mentioned earlier, extensive studies have investigated the electrographic, behavioral, and pathological outcomes of CSE via well-established preclinical animal models. In order to investigate the effect of hippocampal seizures on learning and memory and emotions, animal models with an established face validity have been employed as they recapitulate, in a controlled setting, the clinicopathological outcomes seen in patients with temporal lobe seizures (8,154,155). Of note, due to technical difficulties in producing seizures in animal models, artificial seizure-induction methods are often used as discussed below. Even though these methods successfully reproduce electroclinical seizures, they do not mimic human-like scenarios, and thus respect face validity at the price of construct validity, with a few exceptions of seizure-induction methods like closed-head traumatic brain injury, and hypoxia.

1. Electrical stimulation models

Electrical stimulation models were one of the first studied paradigms employed to investigate seizures. In these models, rodents are stimulated with a standardized amount of electrical stimulation delivered to the temporal lobe, with the hippocampus as the primary target. Hippocampal SE can be induced without the complications of chemically-induced toxicity in chemoconvulsant seizure models. Electrical stimulation models are associated with less severe brain lesions at the site of seizure induction, compared to chemoconvulsant models.

2. Chemoconvulsant seizure models

SE is commonly induced by kainic acid, pilocarpine, lithium-pilocarpine and diisopropylfluorophosphate, due to the enhancement of glutamatergic neurotransmission or blockade of GABAergic inhibition.

a. Kainic Acid (KA) models

KA is a cyclic analog of L-glutamate and an agonist of ionotropic KA receptors. KA induces prolonged excitatory responses in cortical neurons in rats. It can be used as a potent analog of glutamate, inducing robust depolarizations and eventually cell death. KA-induced seizures were proposed as a model with particular relevance to TLE as it shares many of the features of human hippocampal seizures (156), and it has a high affinity for kainite glutamate receptors in the hippocampus (157). KA-induced damage in the hippocampus includes CA1 and CA3 pyramidal neurons and hilar neurons in the dentate gyrus (158) with symmetrical cell loss, where bilateral structures exhibit the same degree of cell death. KA models are simple and easily reproducible, and the resulting SE electrographically resembles the one seen in humans (159). A major drawback of the KA model is the variable sensitivity of rats of different strains, sex, age and weight to KA (160).

b. Other chemoconvulsants

As in the KA model, the pilocarpine-induced SE leads to the development of spontaneous temporal motor seizures and mossy fiber sprouting in the dentate gyrus (161). Lithium-pilocarpine induced SE shares similar features with the high-dose pilocarpine model, such as onset and duration, although they differ in onset severity. Moreover, the mortality rate in the lithium-pilocarpine model is very low compared to the high dose pilocarpine model. However, all chemoconvulsant models can be employed to induce CSE, and have been employed to produce long-term recurrent seizures and behavioral disturbances such as hyperactivity, and deficits in visuospatial navigation and contextual conditioning (8,65,162). These have been used to recapitulate comorbidities of human epilepsies, including learning disorders, aggression, and ADHD (139,163-165).

H. NCSE: a desperate need for valid rodent models

Even though clinical studies aimed at characterizing pharmacological therapeutic approaches to NCSE, there is a paucity of information about the neuropathological and behavioral consequences of this condition. The medical community has relied on a scarce literature to tailor pharmacological approaches and gauge diagnostic and treatment urgency (166). In addition, there are no universally accepted protocols to approach NCSE. The effect of NCSE on neuronal networks are not extensively studied. This is important to know, especially that this condition is clinically underestimated and undertreated. In order to shed light on the yet unclear anatomical and pathological consequences of NCSE on affected neural tissue in patients, there have been various attempts at customizing animal

models (11,156,167-169). Standard animal models of epilepsy such as the pilocarpine model (170,171), the kainic acid model (172,173), the prolonged kindling model (11,174) and the self-sustained temporal status epilepticus model (175), have been used with subconvulsant chemical doses or low electrical intensities in modestly successful attempts at producing models of NCSE. The chemical models of NCSE, similar to those of epilepsy are postulated to exhibit electrographical changes and behavioral symptoms that electroclinically mimic TLE (176,177). In fact, a targeted hippocampal KA delivery has been attempted in pigs with electroclinical outcomes similar to humans (147), but not yet in rats as mentioned in section I.6. Indeed, studies on epilepsy rodents models have been limited to electrical stimulation, or systemic pilocarpine administration, with some success in electroclinically reproducing NCSE (145,146).

Here, we propose to confirm the face validity of a rat temporal NCSE model with targeted intra-hippocampal KA delivery as it is believed to provide a suitable platform for studies on NCSE of the temporal lobe, and its resulting cognitive and emotional behavioral disturbances. Indeed, the temporal lobe houses the amygdalohippocampal circuitry that is involved in emotionally- relevant learning and memory, and when compromised, results in seizure generation, including NCSE.

I. Common behavioral panels for assessing the function of the amygdalohippocampal circuitry in temporal lobe seizure models

Neurobehavioral deficits such as impaired emotionally-relevant learning, and diminished memory, can be studied in a controlled setting where the effect and contribution

of different factors to emotional and cognitive issues can be isolated and analyzed via various molecular and histological techniques. In the TLE models more specifically, the long-term cognitive and emotional behavioral effects of CSE induced by KA or any other chemoconvulsant have been studied using various behavioral tests that mirror human psychiatric and cognitive problems. Short of being able to study feelings directly, investigators have focused on their non-subjective aspects, designing experimental paradigms that provide them with measurable emotional behaviors in rodents for emotionally-relevant learning and memory and adaptive behavior in new environments (178). Consequently, they were able to create a certain parallelism between humans and rodents while respecting the differences in brain circuitry that generates innate versus learned responses to threat (179). In the following are examples of behavioral tests to assess anxiety, depression, and emotionally-relevant learning and memory.

The light dark box test (LDT) and open field test (OFT) are used to assess anxious-like, exploratory and hyperactive behaviors. The forced swim test (FST) is employed to examine struggling behaviors, and depressive-like behaviors. The Morris water maze (MWM) is a test for visuospatial navigation. The modified active avoidance (MAAV) assesses the ability of the rodent to anticipate a potential threat and react accordingly, thus measuring emotionally-relevant learning.

TLE models following SE exhibit depressive-like behavior in the FST, in addition to anxiety-like behavior and hyperactivity in OFT, and deficits in amygdalohippocampal-spatial learning in MWM due to aberrant exploration (65,180,181). In the LDT, following SE, animals spend less time in the lit chamber; have lesser number of transitions between

the two compartments, and a longer latency time to the first entry into the lit chamber, unlike controls. This reflects decreased exploratory behavior, and it has been reported in different epilepsy rodent models with prominent limbic involvement, including hypoxic seizure models, as well as pilocarpine-induced and KA-induced SE models (182-185).

Table 1. The common behavioral panels conducted in our laboratory in order to assess the integrity of the amygdalohippocampal functions in epilepsy rodent models with prominent limbic involvement.

Testing panels	Test description	Test used for	Measurable behavioral responses
Modified active avoidance (MAAV)	In this is novel testing paradigm introduced by our laboratory, rats are placed in a two-chambered box. Electrical foot-shocks are signaled by a tone on one side, and by visual cues on the other side. Shuttling between the compartments prevents shocks	Emotionally-relevant learning of auditory and contextual cues and adaptive shock-avoiding behaviors	Percentage of avoidance behaviors and avoidance latencies (time required to avoid the shock before its onset upon perceiving the conditioned stimulus)
Forced swim test (FST)	Rodents are forced to swim in an enclosed cylinder filled with water and their escape-directed behaviors are observed	Depressive-like behaviors	Time spent immobile, swimming and struggling activities, climbing attempts, latency time to the onset of immobility
Open field test (OFT)	A rodent is subjected to an unknown aversive open arena from which escape is prevented by surrounding walls. It is placed either in the center or next to the walls of the apparatus and its behavior is observed over a predefined session of usually 5 to 10 minutes	Exploratory, hyperactive, and anxiety-like behaviors	Distance traveled, time spent in each zone (central versus peripheral), time spent exploring central objects, latency to enter the central area, time of immobility (freezing), speed in each zone
Light-dark-box test (LDT)	The setup consists of two interconnected compartments, one is black and dim and the other is white, brightly illuminated and contains novel objects. Here we study the conflict between a rodent's exploratory behaviors versus a natural aversion to new lit environments	Anxiety-like behaviors	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)	The maze consists of an open circular pool filled with water that contains a hidden submerged escape platform. Rodents are placed in a designated starting location of the pool and their escape-directed swimming behavior is observed during a defined session of 2 minutes	Visuo-spatial navigation	Escape-directed swimming behavior (escape latency, distance traveled)

The MAAV as described below is a modified instrumental conditioning test that assesses the recognition of auditory emotional cues as well as hippocampal-dependent contextual emotional cues, and the acquisition of learned adaptive shock-avoiding behaviors. This test is one of the main panels used in this work for studying potential functional disruptions in both amygdalohippocampal circuitry- dependent cognitive and

emotional behaviors. and its potential histological, molecular, and behavioral consequences upon chemical (kainic acid) manipulation. It stems from Pavlovian conditioning, or classical conditioning. Classical conditioning, introduced by Ivan Pavlov in 1927, 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 (186,187). The emergence of instrumental conditioning allowed a higher level of investigations that include testing of learned adaptable responses that anticipate and avoid an unconditioned aversive stimulus. This is a form of learning that helps the animal anticipate a potential threat and react accordingly. Its importance lies in the fact that fear of painful foot-shock is a survival-related innate reaction (143,188). Upon multiple associations between a conditioned stimulus (tone or contextual cues) and a non-conditioned stimulus (painful foot shock), the rodents associates between hem and start reacting to the now fear-eliciting CS with freezing responses. Freezing is characterized by a generalized immobility of the subject's musculature, excluding those involved in breathing (189). This species-specific innate fear reaction upon the initial recognition of CS as being a threat is replaced by an acquired avoidance response in instrumental conditioning. Depending on the experimental paradigm, active avoidance may consist of shock avoidance via shuttling between compartments or by stepping over a platform.

CHAPTER II

AIMS AND HYPOTHESES

The three pillars of clinically-practiced medicine are diagnosis, treatment, and prognosis. Knowledge on NCSE is lacking in all three aspects, likely leading to its underdiagnosis, and to delayed treatments, and as importantly a poor understanding of potentially permanent NCSE-related harmful effects on the brain. Moreover, there are no well-established preclinical models to assist us in improving our insight into this condition. Given that NCSE commonly originates from the temporal lobe in adolescents and adults, and in the light of accumulating reports on potential NCSE-related permanent harmful effects, we propose to electroclinically validate a novel peri-adolescent rodent model of NCSE of temporal lobe origin, and study its potential harmful effect on this structure with key cognitive and emotional regulatory functions, as delineated below.

Aim 1: To investigate the electroclinical effect of intra-hippocampal subconvulsive doses of KA in postnatal day 43 (P43) peri-adolescent rats.

Hypothesis 1: Hippocampal KA injections will reliably produce seizure episodes that recapitulate the electroclinical characteristics of NCSE of temporal lobe origin in humans, characterized by the absence of motor manifestations.

Aim 2: To investigate the effects of two episodes of intra-hippocampal subconvulsive dose of KA NCSE in the temporal lobe, 24 hours apart, on potential early cognitive and emotional behaviors.

Hypothesis 2: Two episodes of NCSE of temporal lobe origin will lead to structural and functional disruptions in this structure involved in learning, memory and emotional regulation. Recurrent episodes are induced to create a clinically relevant scenario in this underdiagnosed condition that often comes to medical attention after more than one episode.

Aim 3: To investigate the effects of two episodes of intra-hippocampal subconvulsive dose of KA NCSE in the temporal lobe, 24 hours apart, on potential structural damage by assessing neuronal density and glial fibrillary acidic protein (GFAP), and on alterations in synaptic plasticity proteins, specifically the activity-regulated cytoskeleton-associated protein (Arc) and synaptophysin (Syp) by immunohistochemistry.

Hypothesis 3: Recurrent episodes of NCSE of temporal lobe origin will result in alterations in markers of hippocampal synaptic plasticity known to be modulated by hyperexcitability (80,81,92,93), and in potential hippocampal neuronal damage.

CHAPTER III

MATERIALS AND METHODS

A Animals and experimental design

The Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut approved all experiments. Forty-five male Sprague Dawley rats housed in a temperature-controlled room and maintained on a 12-hour light-dark cycle with permanent access to food and water were used in this study. Rats were subjected to one or two episodes of NSCE or sham manipulated as described below (section III.3.) Following NSCE, rats were subjected to a battery of behavioral testing panels and then sacrificed at postnatal day 50 (P50) for histological analyses, including hippocampal pyramidal neuronal density, GFAP in astrocytes, in addition to the hippocampal protein levels of markers of synaptic plasticity Arc, and Syp.

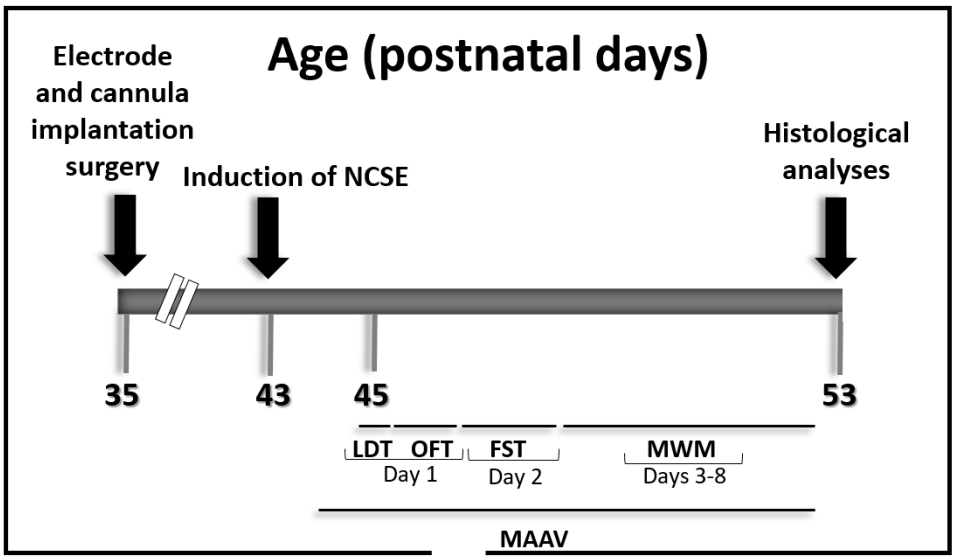

Figure 8. Experimental design of the project. Following an electrode and cannula implantation surgery at P35, rats are granted one week to rest, and then they are placed in the EEG system and NCSE is chemically induced. Rats are then assigned to undergo one of the two behavioral sets depicted in the figure. The first behavioral paradigm includes a series of tests, which are the light-dark box test (LDT) and the open-field test (OFT) on the first day, followed by the forced swim test (FST) on the second day, and concluding with the Morris water maze (MWM) performed over 6 days. The second behavioral paradigm consists solely of the modified active avoidance test (MAAV) performed over 8 days in a parallel cohort of rats. Rats that underwent either behavioral paradigms are sacrificed at P53 for histological analyses.

B. Surgical EEG electrodes/cannula implantations and EEG recordings

Epidural electrode surgery was performed at P35. Rats were anesthetized with an intramuscularly administered mixture of ketamine (60 mg/kg), xylazine (6 mg/kg), and acepromazine (1.25 mg/kg). Once appropriate anesthesia was achieved (lack of signs of pain in response to toe pinching), the rat was placed on a pad, and the hair was shaved with a trimmer from the flat of the nose between the eyes down to the neck. The rat's head was then tightly secured on a stereotaxic frame. Lubricating eye ointment was then applied to prevent drying out and possible eye irritation from the disinfectants. Under sterile conditions (application of iodine then ethanol), an incision was made, and the skull was exposed using a retractor. Five small 1.4 mm holes were made in the skull with a high-

speed drill in order to place five epidural screw electrodes. These included left and right frontal electrodes (2 mm anterior to, and 3 mm lateral to the bregma), left and right parietal (5 mm posterior to, and 3 mm lateral to the bregma) and one anterior midline reference electrode (6 mm anterior to the bregma) (Figure 9) based on the Sherwood and Timiras Atlas of the developing rat brain.

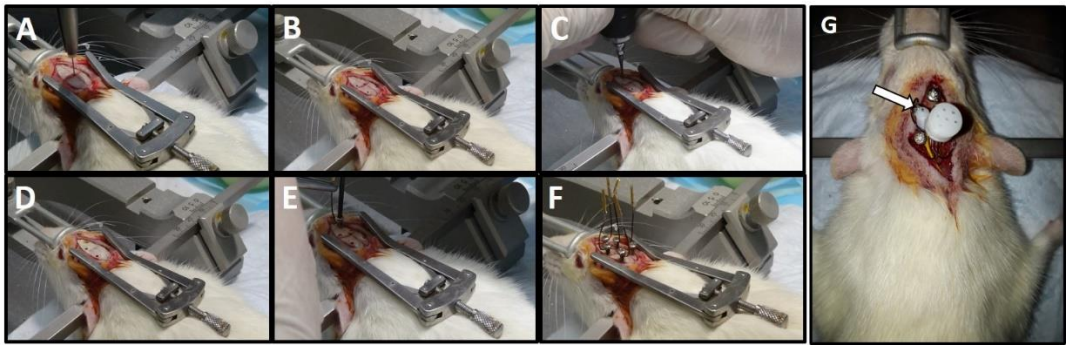

Figure 9. Electrode implantation surgery. Panel A. Once the skull is exposed, cleaned and dried, the bregma is identified with the stereotaxic arm and its antero-posterior and lateral coordinates are recorded. **Panel B.** The coordinates of the five reference and sampling electrodes are then calculated based on the Bregma's coordinates, and their locations are marked one by one by moving the stereotaxic arms to the desired coordinates. **Panels C and D.** After marking the drilling location of the electrodes, five small holes are made with a high-speed drill held in the vertical position above the skull's surface. **Panel E.** The screw electrodes are inserted using a screw driver. **Panel F.** Shown are the two frontal and two posterior parietal electrodes, along with one reference electrode. **Panel G.** Shown here is the assembled electrodes into the pedestal to the right of the cannula (white arrow) that is placed above the left hippocampus.

The electrode wires were then inserted in a 6-channel pedestal (Plastics One, USA) along with a 6th socket attached to a free wire placed under the skin of the neck serving as the ground electrode. An additional hole was made to implant intra-hippocampal guide cannula (2.6 mm in length) that targets the CA1 region of the hippocampus (2.4 mm posterior to, and 2 mm lateral to the bregma, internal cannula length is 3 mm). The wires and screws, along with the cannula's plastic pedestal were then covered with dental cement to give the "headset" its final shape. Neomycin powder was applied to the exposed skin edges in contact with the dental cement to prevent

postoperative infections. Rats were transferred to customized single-animal EEG cages. Analgesia regimens with paracetamol were administered for 3 days postoperatively (1mg/ml in drinking water). Following a 5-day post-surgical recovery period, rats were attached to the EEG customized system and EEG recordings were initiated in order to monitor baseline electrical activity for 24 hours prior to KA injections (Xltek, Natus Medical, USA) (Figure 10).

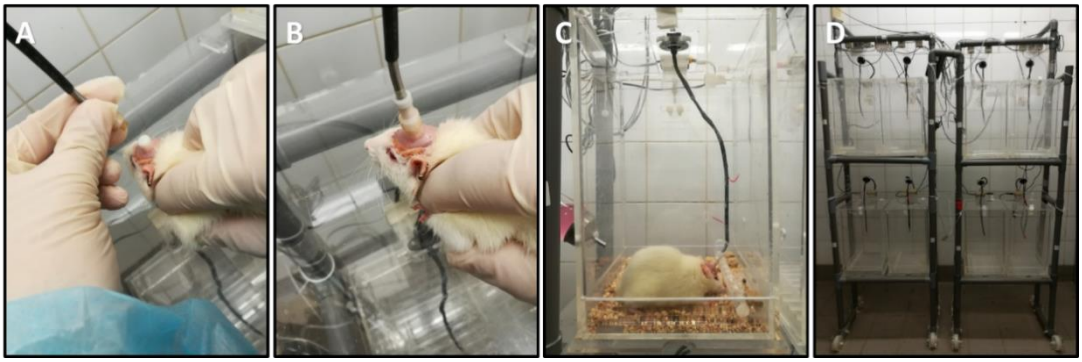

Figure 10. Initiating EEG recordings. Following a 3-5 day post-surgical recovery period, the rat's headset is attached to the EEG cable (**Panel A**). Rats are placed in special cages equipped with a commutator that allows the EEG cable to rotate, and thus accommodating their movements (**Panel B** and **C**). Sown in **panel D** is the full setup of the EEG system, made with non-conductive material to prevent any external electrical noise, hence providing and EEG representation with a high signal-noise ratio.

C. NCSE induction via intra-hippocampal cannula KA injections

Since NCSE in adolescents is often of temporal lobe origin, we performed hippocampal NCSE induction experiments in the peri-adolescent age (P35-45). One or two episodes of NCSE were induced with one or two (24 hours apart) intra-hippocampal KA injections respectively, 0.00625 μg each, dissolved in normal saline, in EEG-monitored peri-adolescents (P43) male Sprague Dawley rats. The used specific subconvulsive dose of KA is the result of multiple trials in our laboratory, and resulted in an electrographic pattern

that recapitulates the electroclinical characteristics of NCSE of temporal lobe origin seen in adolescent and adult humans.

Rats were divided into the following three groups:

1. ***RKA group:*** two episodes of NCSE were induced by two 24-hr apart KA injections (0.00625 µg in normal saline). (n=19)
2. ***SKA group:*** one episode of NCSE was induced by one KA injection (0.00625 µg in normal saline). This group was sham manipulated for the second KA injection 24 hours post the first injection. (n=4)
3. ***Control group:*** sham manipulation was performed with cannula and electrode implantation, and two 24- h apart injector insertion in the cannula. We chose not to inject saline, as volume in and of itself may produce hyperexcitability or potentially brain lesions. Hyperexcitability and brain lesions (irrespective of induction mode), are parameters that we want to study in the RKA and SKA groups but not in the control group.

Live behavioral monitoring of rats was performed over 2 hours after each KA injection; however, EEG recording was carried out for 48 hours post the first KA injection.

D. Cognitive and emotional behavioral panels

Rats belonging to the three groups described above were first subjected to the modified active avoidance (MAAV) behavioral test (described below) one day post the second KA injection.

Based on the interesting results obtained from this test, RKA and control groups were tested in a battery of additional panels (still ongoing to reach a power of 10-15 rats per group) tailored to the amygdalohippocampal circuitry, and performed from the least to the most aversive in the following order: Light dark box (LD), Open Field test (OFT), Forced Swim test (FST), and Morris Water Maze test (MWM).

1. The modified active avoidance (MAAV) test

This test was developed at our laboratory to simultaneously assess contextual and auditory cued conditioning, as well as learning of adaptive shuttling behaviors. The MAAV test is 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) and infrared beam sensors that detect transitions between the chambers.

Rats at (P45) were subjected to the MAAV test 24 hours post the second KA injection. The test consisted of one habituation day followed by 6 training days and one retention test day. During habituation, the rats were allowed to acclimate to the shuttle box compartments for 5 minutes, where the walls of both compartments were covered with white foam plates. 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 (Figure 11). 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).

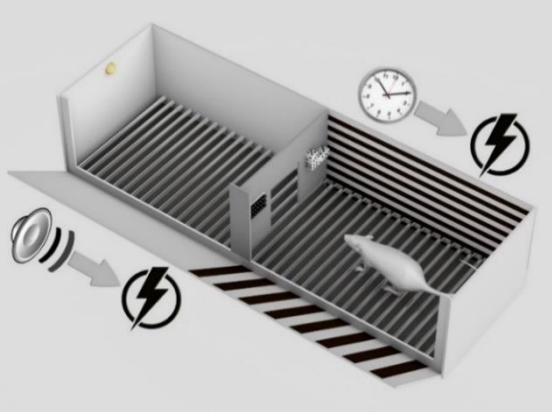

Figure 11. Schematic design of the modified active avoidance test (MAAV). We developed this test by modifying the shuttling box in order to simultaneously test conditioning to contextual and auditory stimuli. In addition to testing the recognition of auditory emotional cue and hippocampal related "visual relational" emotional cues, the test assesses learning of adaptive shock-avoiding shuttling responses that replace innate fear responses (freezing).

2. Light dark box

P45 rats were allowed in a 5-minute session to explore freely a novel environment composed of two different compartments using the shuttle box described in section III.4.1. The left side is lit and has white walls with visual cues (dices and beads). The right side is dark, with black walls. Rats were initially placed in the dark compartment (Figure 12). After testing, the apparatus was cleaned with unscented detergent then 70% ethanol and allowed to dry before the next rat was tested. The LDT outcome parameters (total time spent in each compartment and the number of transitions) were obtained using Graphic State 4 software (Coulbourn Instruments, Harvard Apparatus, USA). The latency to the first entry into the lit compartment was obtained by analyzing the recorded videos using SMART video tracking 3.0 software (Panlab, Harvard apparatus, USA).

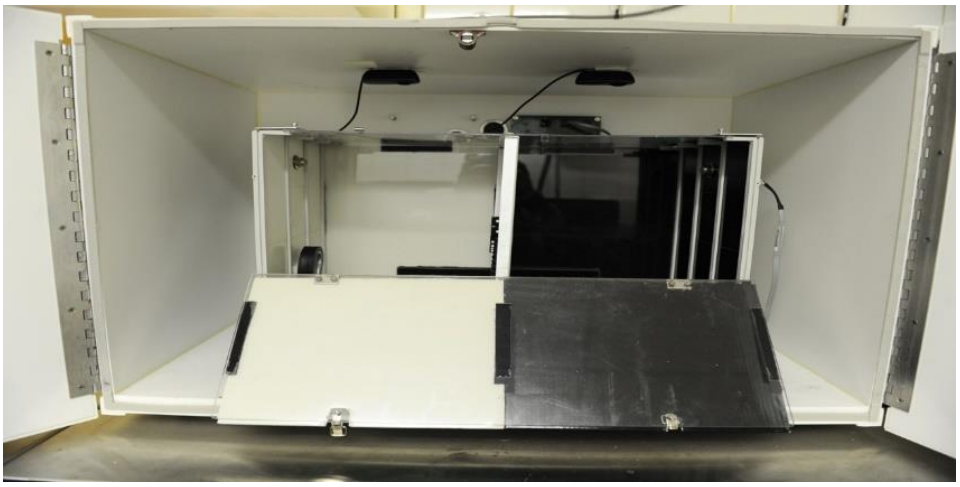

Figure 12. Setup of the light-dark box. The right chamber is surrounded by white walls and it is lit during the course of the experiment. The left chamber is surrounded by black walls and it is kept dark.

3. Open field test (OFT)

The OFT was conducted over three sessions, three minutes each, performed over one day using an opaque plexiglas square field (W 80 cm, L 80 cm, H 40 cm) (Figure 13). On the first testing session, a single small object (cube), was placed in the middle of the field's floor, then on each of the two subsequent sessions, a new object was added (a small ball, then a small bottle). Rats were placed individually in the nearest corner to the most recently added novel object on a given session. The floor surfaces and walls of the apparatus were cleaned with unscented detergent then a 70% alcohol solution between animals. The rats' movements were video-recorded then analyzed using SMART video tracking 3.0 software (Panlab, Harvard apparatus, USA).

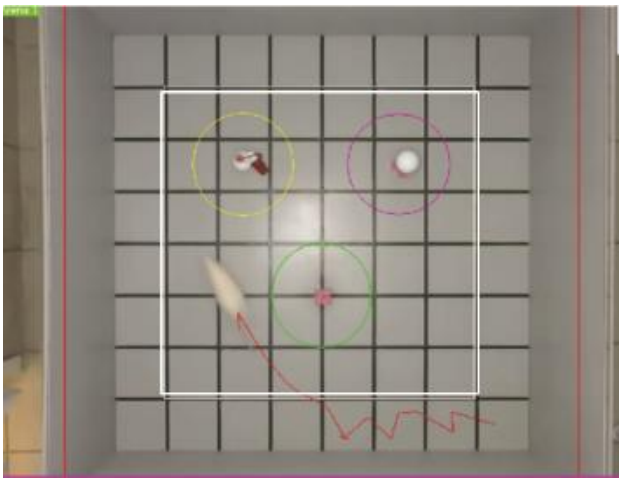

Figure 13. The Open Field test. The rat is placed in an open area and it is left to explore its surroundings. Shown here is a session where all objects are placed, and the rat freely roams throughout the field.

4. Forced swim testing (FST)

The FST consisted of a 7-minute session. Transparent plexiglas cylinders (50 cm in height, 20 cm in diameter) were filled to a depth of 25 cm at 25°C (Figure 14). Rats were placed in the cylinders and their swimming behaviors were video recorded (indicated by the red arrow in Figure 14) then analyzed for struggling and immobility using the SMART software (Panlab, Harvard apparatus, USA). Immobility was defined as the minimal limb movement required to stay afloat. 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. The Forced Swim test (FST). Rats are placed inside water-filled cylinders, and kept for a seven-minute session in order to video record (indicated with the red arrow) their activity levels for later interpretation.

5. Morris Water Maze (MWM)

A dark-blue circular plastic pool, 150 cm in diameter and 80 cm in height (Coulbourn Instruments, USA) was filled to a depth of 30 cm with 25°C water (Figure 15). On the first training day, rats were first allowed to swim for two minutes freely, with no escape platform, in order to habituate to the testing environment. Then during the spatial acquisition trials on days 1-5, an "invisible platform" was placed two cm below the water surface. Rats were placed in the pool, and if they failed to find the platform in two minutes, they were placed on it for 30 seconds by the operator. Four daily trials were performed with a 30-second inter-trial resting period, and four immersion landmarks that were equidistant to the platform and their sequence was changed every day. On testing day 6, the probe trial was performed to assess retention of spatial navigation. The platform was removed, and rats were immersed in water at a novel starting position opposite to the quadrant where the platform was located, and were allowed to swim freely for two minutes. On the same day,

in order to assess motor and visual functions, rats were allowed to swim to a visible platform with four trials per rat. The MWM experiments were video recorded and analyzed with the automated SMART tracking video software.

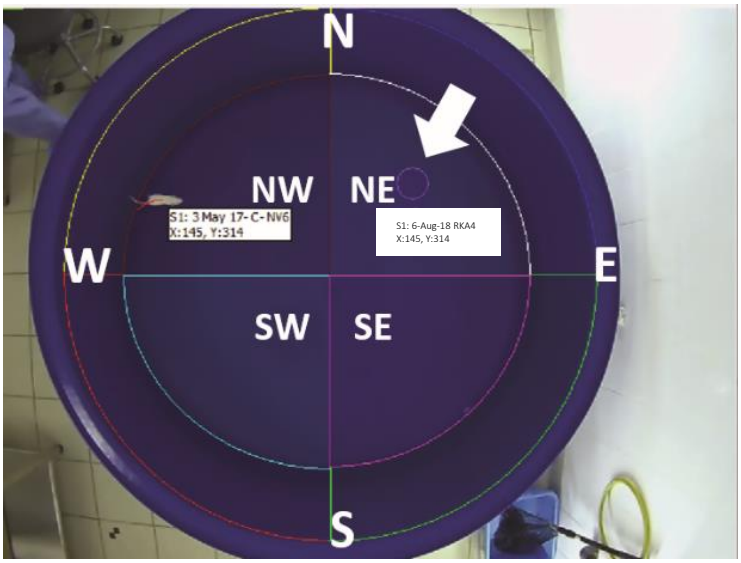

Figure 15. The Morris water maze test during the acquisition days. An invisible platform (white arrow) is placed below the water surface, and the rat is placed in the pool over four sessions from four immersion landmarks (NW,NE,SW,SE) that were equidistant to the platform and their sequence was changed every day. (NW: north-west; NE: north-east; SW: south-west; SE: south-east)

E. Euthanasia and cardiac perfusion surgery for histological and immunohistochemistry studies

In order to perform various microscopic examinations on brain slices, brains were perfused with 4% depolymerized paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (pH=7.4). The procedure starts with general anesthesia using the same anesthetic regimen described in the electrode surgery above. Once anesthesia is achieved (unresponsiveness to noxious stimuli), the animal was be placed on its back. Pins were used to restrain the limbs to prevent accidental or fluid-induced shifting during the procedure. An incision in the abdomen was made just below the diaphragm. The diaphragm was then

be removed to access the rib cage. Two vertical cuts were made along the sides of the rib cage, allowing it to be lifted, exposing the heart. A needle was then inserted into the left ventricle and (PBS) (1X) solution was pumped slowly into the animal. Immediately after insertion of the needle in the left ventricle and just before starting the pump, the right atrium was cut open to allow fluid to drain. Once all the blood has been flushed from the animal, the brain was fixed by PFA, brains were harvested and embedded in paraffin. Coronal sections of 8 µm in thickness were obtained for histological and immunohistochemistry analyses, namely hippocampal neuronal density quantification and for hippocampal Arc, and Syp staining.

1. Molecular markers of synaptic plasticity (Arc and Syp) via immunohistochemistry

Immunohistochemistry was performed on brain sections from each group. Slides were first deparaffinized with xylene. Slides were incubated for 60 minutes in sodium citrate buffer for antigen retrieval, and then washed in PBS. Following blocking with 10% Normal Goat Serum (NGS), slides were incubated overnight with primary antibodies solution for Arc or Syp using the following antibodies: Arc (sc-17839; dilution 1:500), and Syp (sc-12737; dilution 1:250) both obtained from Santa Cruz Biotechnology. Slides were then washed and incubated with Horse-Raddish Peroxidase (HRP) conjugated secondary antibodies obtained from Santa Cruz Biotechnology (sc-516142; dilution 1:100). Slides were washed, stained with DAB, counterstained with hematoxylin.

2. Hippocampal neuronal count on Hematoxylin and Eosin (H&E) stained brain sections

Following deparafinnization, slides were stained with H&E. Nine sections from each brain were chosen based on the location of the cannula. The identification of the location of the cannula was performed by visual inspection of the sections. Subsequently, the slides were imaged using uSCOPE (uScope MXII, USA) machine, and then cell count was performed manually using the ImageJ program. An investigator blinded to the treatment groups performed the counting of pyramidal neurons in the CA1-CA3 hippocampal region.

3. GFAP staining

Astrocytes are specialized glial cells that contribute to the maintenance of the blood-brain barrier, regulation of extracellular glutamate levels, vascular reactivity, and protection from reactive oxygen species (190,191). These cells react to neuronal damage resulting from physical or chemical insults, and exhibit altered morphology, featuring an over expression of GFAP. This is of particular interest in seizures associated with glial scar tissue including post-traumatic epilepsy (192,193) and kainite seizure models of TLE (194,195). The sections were deparaffinized and rehydrated with decreasing concentrations of ethanol. The sections were then immersed in 0.3% H_2O_2 to block endogenous peroxidase, and then incubated with 1.5% normal goat serum. This was followed by incubation with primary antibodies overnight. The sections were then incubated with biotinylated goat anti-rabbit secondary antibody obtained from Santa Cruz Biotechnology

Laboratories. The immunoreactive signals were amplified by incubation with the avidin-biotin peroxidase complex. Finally, the sections were counterstained with hematoxylin and imaged using uSCOPE.

F. Statistical analyses

Statistical analyses were performed using Prism 7 (GraphPad Software, USA). One way analysis of variance (ANOVA) in conjunction with the post hoc Fisher least significant difference (LSD) test was used to analyze LDT, OFT, and MAAV retention data, and MWM visual platform test. Two-way ANOVA with repeated measures in conjunction with the post hoc Fisher LSD test was used to analyze data obtained from the FST, the MAAV training test, and from the MWM spatial acquisition testing, in order to assess the effect of the day of attesting as well as that of the intervention.

CHAPTER IV

RESULTS

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

Following multiple dosing trials, we found that a specific low dose of KA (0.00625 µg) delivered via stereotaxically-placed hippocampal cannulas above the CA1 area, consistently induced prolonged non-convulsive electroclinical seizures that recapitulates its human counterpart in 90% of experimental P43 rats. This was evidenced by seizure activity on EEG accompanied by behavioral changes that are similar to the ones seen in humans with temporal NCSE. The ictal EEG changes consisted of rhythmic fast spikes, spike waves (Figure 16), in additional to occasional poly spikes. Based on the Racine scale, the behavioral changes of Racine scale 1 and 2 included behavioral arrest, decreased locomotion, salivation and repetitive chewing, wet dog shakes, and staring (Table 2) that clinically recapitulate temporal NCSE in humans. Moreover, there was no progression to forelimb clonus or rearing and falling, which reflect Racine scale 3 or more, and not NCSE. The duration of electroclinical seizures lasted on average 63.69 (±13.55 min) after each injection in the SKA (62.2 min ± 15.13 , n=4) and the RKA (75.3 min ± 4.43 on day1; 51.2 min ± 8.13 min on day 2, n=15) groups. The average latency to NCSE onset post-KA was comparable between the SKA (24 minutes) and RKA (27.7 minutes on day 1, and 31.6 minutes on day 2).

Table 2. The Racine scale. Table showing different scores on the Racine scale and their corresponding behavioral manifestations.

Score on Racine scale	Behavioral manifestations
1	Behavioral arrest, oromotor automatisms
2	Head nodding, facial clonus and shivering
3	Forelimb clonus
4	Forelimb clonus, rearing and falling
5	Generalized tonic-clonic activity, death

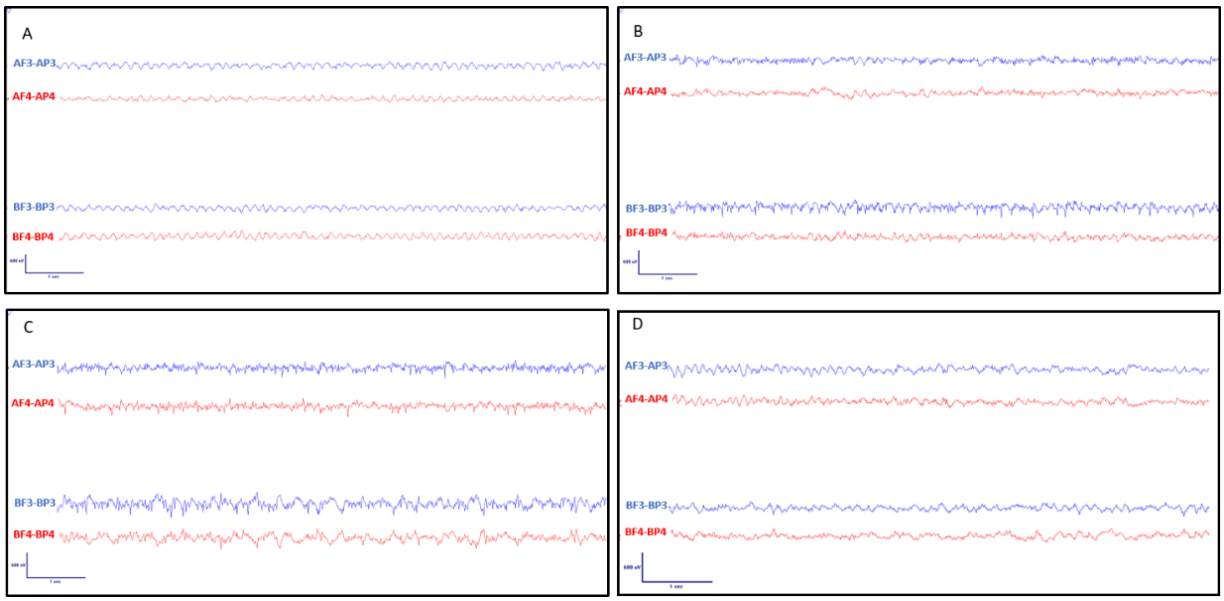

Figure 16. EEG patterns of NCSE evolution shown in longitudinal bipolar montage. Shown is the tracing from two rats (rat A and rat B), each with four sampling electrodes (F3: left frontal, F4: right frontal, P3: left parietal, and P4: right parietal). **Panel A.** baseline electrical activity in two rats before KA-induced NCSE. **Panel B.** Focal NCSE onset in both rats. Rat A shows bilateral fast activity more prominently seen on the left side. Rat B shows left rhythmic fast spikes over the left side with a contralateral field into the right side. **Panel C.** Generalization of NCSE post-KA injection. **Panel D.** NCSE offset; partial return to baseline electrical activity as evidenced by the theta activity intermixed with slow waves.

B. Effects of one or two NCSE episodes on contextual and auditory learning and memory

We investigated amygdalohippocampal-dependent learning of emotional cues and adaptive shock-avoiding behaviors in the MAAV. In the 6-day training phase, rats of all three groups gradually learned to avoid tone-signaled electrical foot-shock in the left compartment, and context-cued shocks in the right compartment. The RKA group showed similar learning curves of tone-signaled and context-cued shock-avoidance compared to controls; however, they eventually plateaued in learning on the last test day in both retention of contextual learning and auditory learning compared to the CTR group (Figure 17). Counter-intuitively, the SKA group showed a tendency towards an improved rate of learning of shock-avoiding behaviors of both tone-signaled and context-cued electrical foot-shocks (Figure 17). This unexpected enhancement in learning was also observed in the retention day, where the SKA rats showed a higher retention rate compared to both RKA and controls.

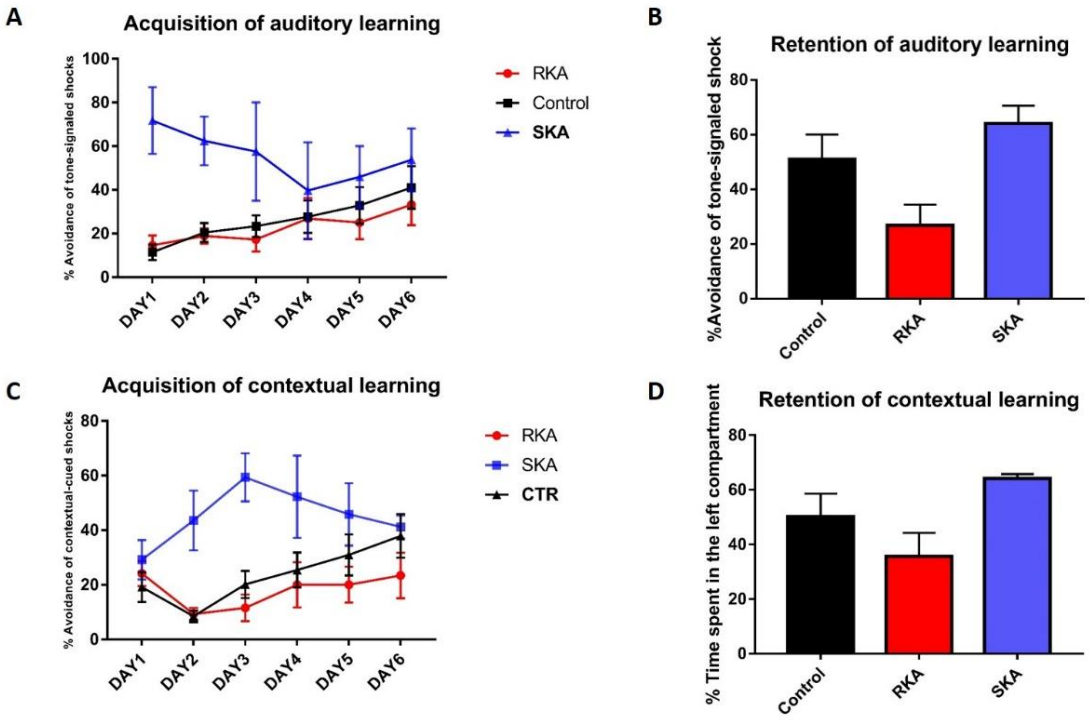

Figure 17. Percentage of context-cued (panels A and B) and tone-signaled (panels C and D) avoidance of electrical foot shocks (CTR n=18; RKA n=15; SKA n=4). Panel A. Acquisition of tone-signaled electrical foot shocks. RKA rats showed a similar learning curve compared to the controls. However, SKA rats had a better learning curve on the first 3 days compared to the other groups. Despite the low number of rats in this preliminary data set, this effect reached statistical significance (ANOVA $p<0.05$, SKA compared to RKA and CTR on all 3 days). **Panel B.** Retention of auditory learning on the last day of the test. The RKA group was less successful in avoiding tone-signaled shocks (33.1% ± 6.2%) compared to controls (51.1% ± 8.8%) and to SKA (64.2% ± 8.9%), which were comparable. **Panel C**. Acquisition of context-cued electrical foot shocks. RKA rats showed a similar learning curve compared to the controls. Interestingly, SKA had a better learning curve on days 2, 3 and 4 compared to RKA and CTR. Despite the low number of rats in this preliminary data set, this effect reached statistical significance (ANOVA, $p<0.05$, SKA compared to both CTR and RKA on days 2, 3, and 4). **Panel D.** Retention of contextual learning on the last day of the test. Contextual learning assessed by the time spent outside the right compartment, revealed retention deficits in the RKA group compared to the other groups (time spent in the left "non-threatening" compartment: 35.6% ± 8.7% for RKA, 50.2% ± 8.4 for the controls (one-way ANOVA $p>0.05$). Interestingly, the SKA group showed a higher retention rate with more time spent outside the threatening compartment (64.0% ± 1.9%). (SKA: single NCSE episode; RKA: two episodes of NCSE; CTR: sham manipulated controls).

Moreover, upon exposure to a continuous tone for two minutes without shock administration, the RKA rat exhibited a maladaptive increase in freezing compared to controls, whereas the SKA rats showed little or no freezing behaviors (Figure 18).

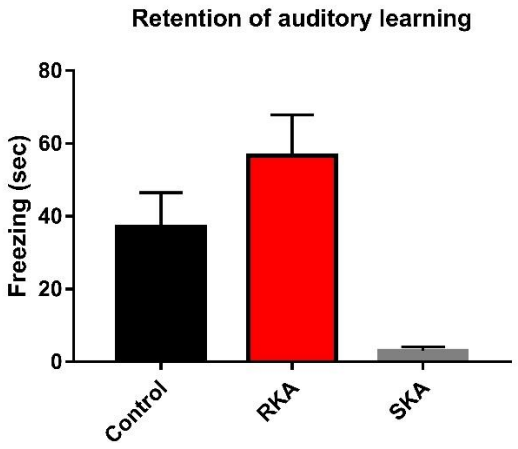

Figure 18. Freezing. Freezing is quantified to assess the retention capacities of the rat. Compared to the controls (37.1 sec ±9.4 sec), RKA (56.6 sec ±11.3 sec) rats showed a higher rate of maladaptive freezing, and SKA (4.4 sec ±1.1) exhibited little to no freezing.

C. Anxiety-like behaviors, exploratory behaviors, and hyperactivity in the LDT and OFT

We assessed the effect of two NCSE episodes on activity level, exploratory tendencies, and anxious-like behaviors in closed (LDT) and open environments (OFT). In the LDT, both RKA and control groups were comparable in the percentage of time spent in the lit chamber of the box, and the latency to first transition from the dark to the lit chamber (Figure 19). However, two episodes of NCSE resulted in a decrease in exploratory tendency in this preliminary data set (n=4 per group) as evidenced by a decreased number of entries into the lit chamber (Number of entries: CTR=5.5 ± 1.7, RKA=3.5 ± 0.6, Figure 19), hinting to an anxiety-like behavior

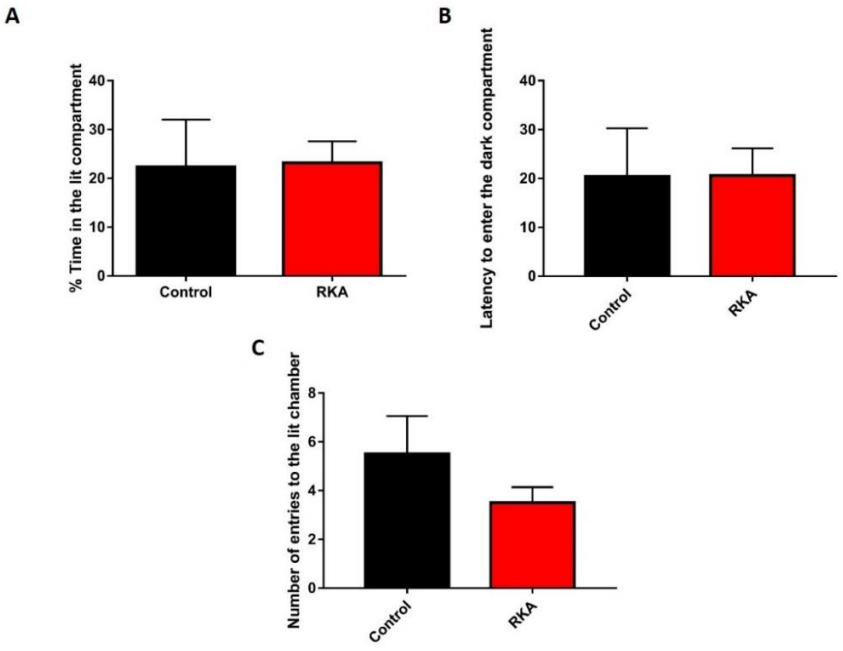

Figure 19. Light-Dark box test (n=4 per group). Panel A. Percentage time spent in lit compartment was comparable between all groups (p>0.05 student T test). **Panel B.** Latency to first entry to the dark compartment was also comparable between both groups. **Panel C.** Number of entries to lit compartment. The CTR group had a higher number of entries compared to the RKA group (CTR=5.5 ± 1.7, RKA=3.5 ± 0.6). (RKA: two episodes of NCSE; CTR: sham manipulated controls)

In the OFT, we have so far not detected differences in the total distance traveled by the different groups on any single session or in the cumulative total distance traveled over the three testing sessions (distance travelled: CTR= 8785 cm ± 482, RKA=7223 cm ± 650, Figure 20). All groups spent comparable durations in the outer zone and in object exploration in the center of the open field on each session and cumulatively over the 3 sessions.

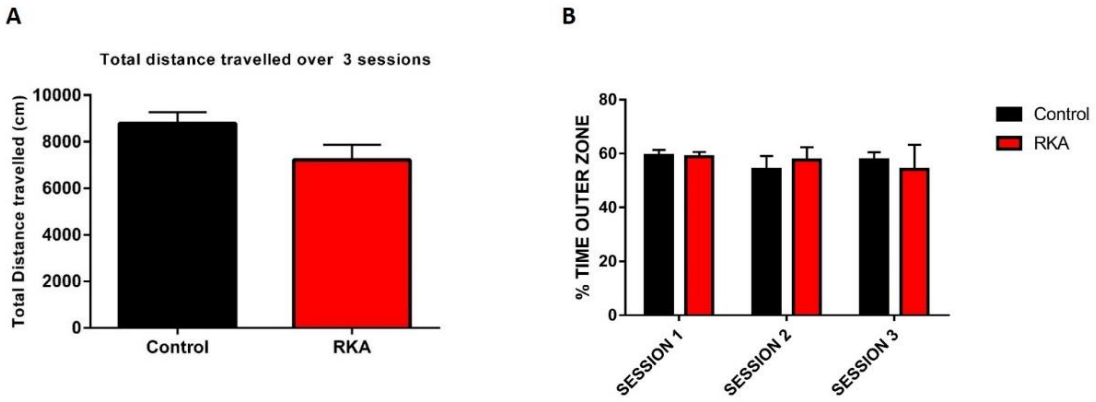

Figure 20. The Open Field test. (n=8) Panel A. The total distance travelled was comparable between the RKA group (7223 ± 649.6) and controls (8785 ± 481.8). (RKA: two episodes of NCSE; CTR: sham manipulated controls). **Panel B.** The total time spent in the outer zones of the Open Field was comparable between RKA rats and controls in all three sessions.

D. Depressive-like behaviors in the FST

We also assessed the effect of two episodes of NCSE on struggling and depressive-like behaviors in the FST. We found that struggling behavior of the RKA group diverges from that of the control rats at the 5th minute of the test. Indeed, in the RKA group, the immobility percent increased steadily from 20% at minute 4 to almost 50% in the last minute of the test as opposed to control rats that kept struggling with less than 25% immobility throughout the test (RKA=48.7%± 7.4%, CTR=21.4%± 2.6%, Figure 21). This decreased struggling in the RKA group was interpreted as a depressive-like behavior as it is not due to hypoactivity based on the OFT outcomes.

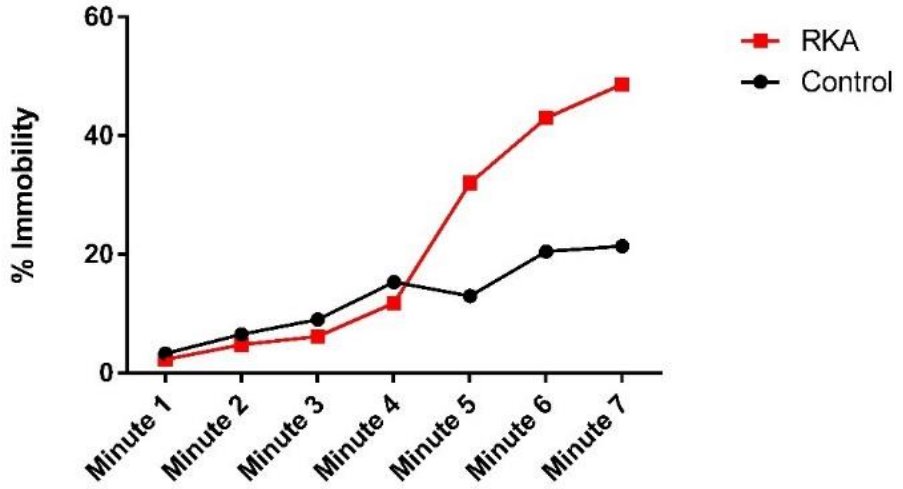

Figure 21. Percentage of immobility stratified by minute in the Forced Swim Test (n=8). The RKA group had a decreased rate of struggling as shown by the immobility curves. CTR and RKA rats had a comparable immobility curve until minute 4 of the test where they diverged. (RKA: two episodes of NCSE; CTR: sham manipulated controls).

E. Visuospatial Navigation in the MWM

In order to evaluate visuospatial learning following two episodes of NCSE, rats were subjected to the MWM test. Escape latencies were comparable between the two groups during the 5-day spatial acquisition training (Figure 22). These preliminary results (n=4) suggest the absence of a decline in hippocampal-dependent visuospatial learning in the peri-adolescent age following two NCSE episodes.

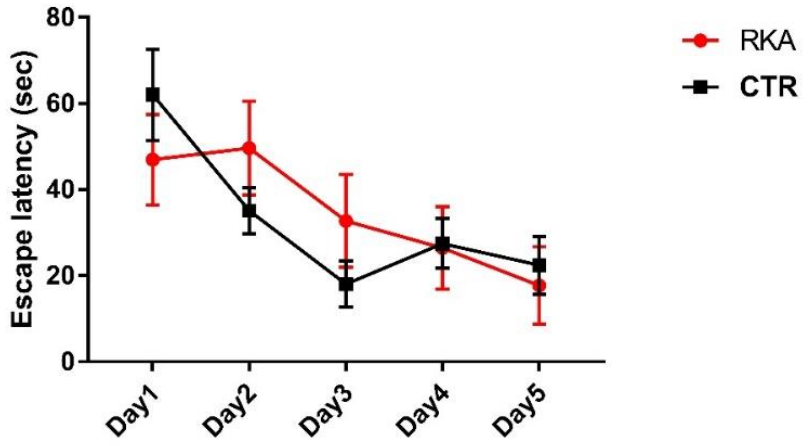

Figure 22. Escape latencies during the acquisition phase of the Morris water maze. RKA rats and Control rats showed comparable escape latency curves throughout the five acquisition days. (RKA: two episodes of NCSE; CTR: sham manipulated controls).

F. Histological and structural analyses

Since we are aiming at characterizing the mechanistic underpinnings of potential NCSE effects on the brain, we assessed hippocampal pyramidal neuronal cell counts (Figure 23). There were no statistically significant differences in hippocampal pyramidal cells numbers between RKA and CTR in the left hippocampus where the cannula was inserted (Pyramidal neuronal counts in CA1-CA3 layers: RKA= 13.8 cells/mm ± 1.2, CTR= 14 cells/mm ± 0.9, student T test, $p > 0.05$, Figure 24). These results indicate that there is no overt cell damage after two episodes of NCSE and probably there will be no cell loss after one episode of NCSE. In order to further assess potential NCSE-related morphological changes, we performed GFAP immunostaining for reactive astrogliosis. All groups showed similar patterns of GFAP expression throughout the area of interest in the hippocampus (Figure 25).

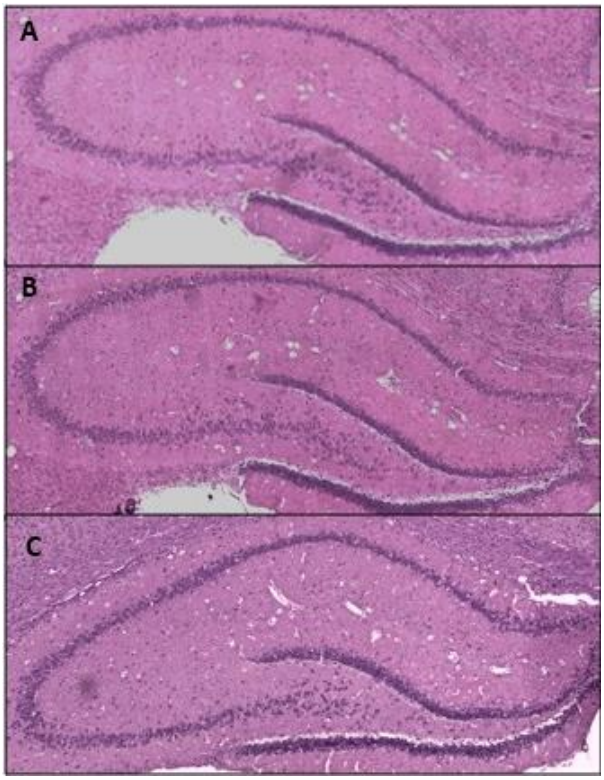

Figure 23. H&E staining of the cannulated hippocampus. Shown are illustrative images of the controls (Panel A), RKA (Panel B), and SKA (Panel C). No overt cell damage was detected in any of the treated groups compared to the control group. (RKA: two episodes of NCSE; SKA: one episode of NCSE; CTR: sham manipulated controls).

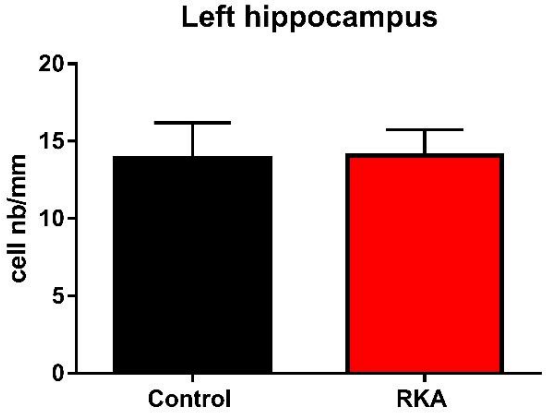

Figure 24. Pyramidal neuronal cell counts in CA1, CA2 and CA3 layers (n=4 per group). The number of hippocampal pyramidal neurons was comparable between RKA and CTR rats ($p>0.05$). (RKA: two episodes of NCSE; CTR: sham manipulated controls).

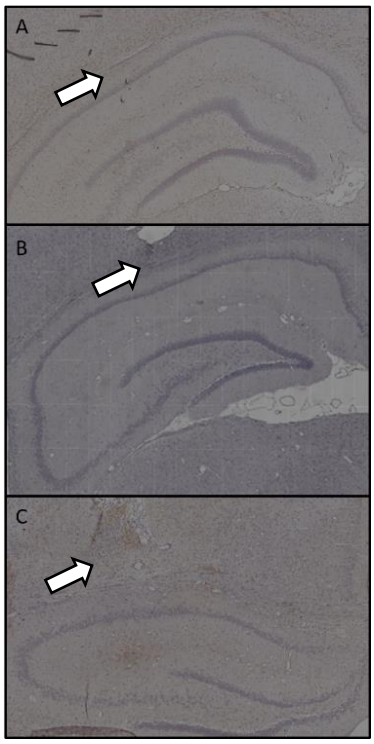

Figure 25. GFAP immunostaining. Controls, RKA and SKA groups (Panels A, B and C respectively), showed similar patterns of GFAP expression around the CA1 region and the dentate gyrus. Of note, the pronounced astrogliosis (white arrows) corresponds to the cannula site, and is likely attributable to mechanical injury.

G. Molecular markers of synaptic plasticity post-MAAV

To investigate the effect of NCSE on synaptic plasticity, staining for Arc and Syp proteins was performed in each group (n=3 per group). Arc is an immediate early gene required for consolidation of memory, exhibiting a shift from basal levels after some environmental changes and post-hippocampal dependent tasks such as fear conditioning and spatial-navigation tasks. Arc expression was examined in all three groups (RKA, SKA and CTR) post-MAAV. Neurons that that exhibited intense labeling were designated ‘’Arc-positive’’ cells. Compared to the controls, a larger number of such cells was detected in the SKA and RKA groups, with a more robust expression in the SKA group (Figure 25). Arc-

positive cells were located in the hilar and dentate regions of the hippocampus of these rats. Corroborating with the Arc patterns, immunostaining for Syp also revealed increased protein levels in the RKA and SKA groups compared to the controls. In addition, compared to RKA, Syp expression was distributed in a more confluent pattern the hippocampus of SKA rats (Figure 26).

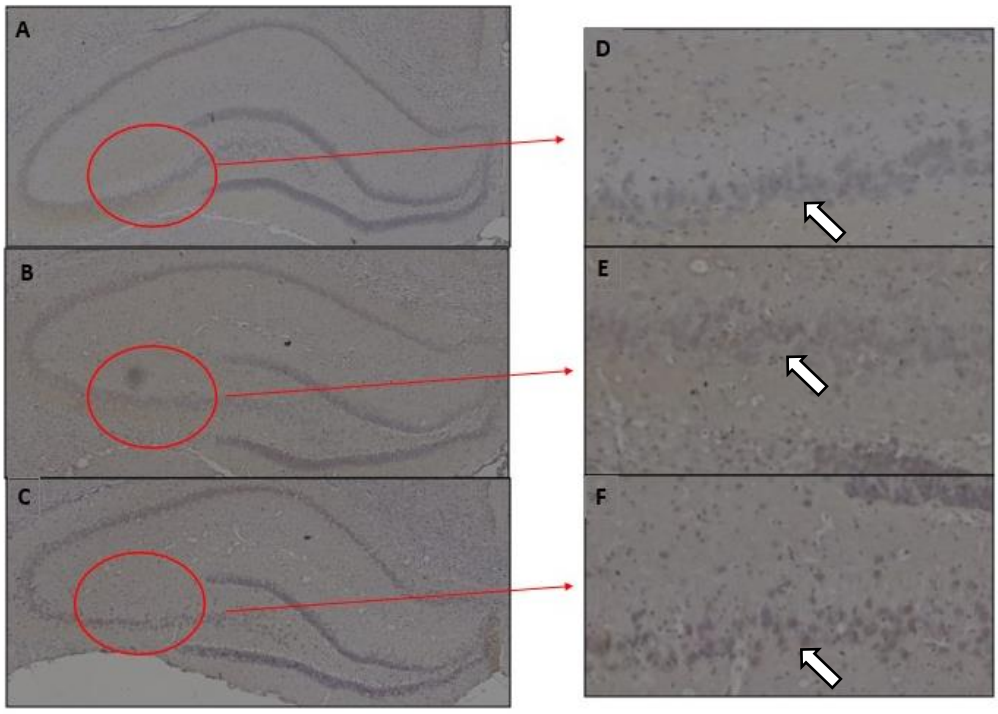

Figure 26. Arc staining on CTR (A), RKA (B), and SKA (C) groups. Arc positive cells were located in the CA4 region of the hippocampus, whereby RKA and SKA rats (**panels E and F respectively**) showed a higher number of Arc-positive cells (white arrows) compared to the controls (**panel D**). (SKA: one episode of NCSE; RKA: two episodes of NCSE; CTR: sham manipulated controls, Arc: activity-related cytoskeletal protein).

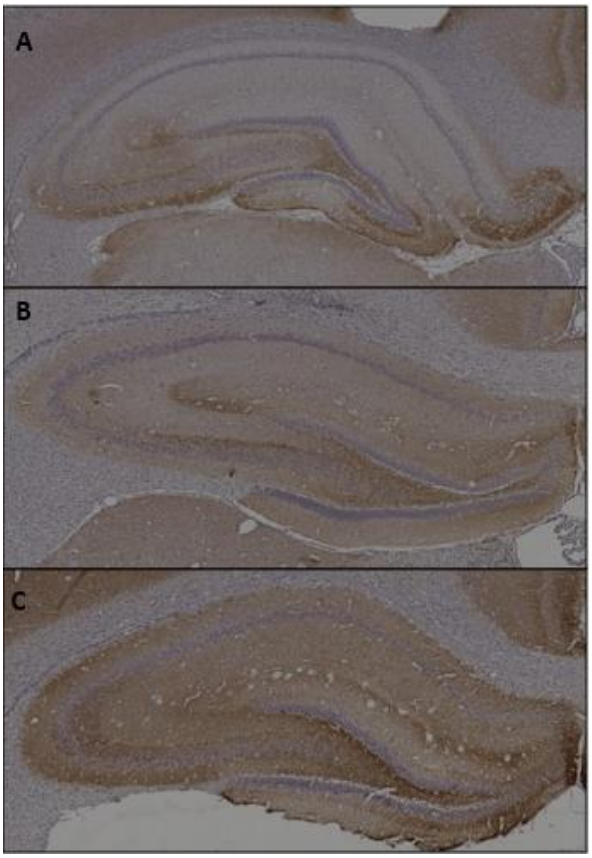

Figure 27. Syp staining on CTR (A), RKA (B), and SKA (C) groups. Syp expression was located mainly in the dentate gyrus and in the CA4 hippocampal regions, and was more prominent in the RKA and SKA groups compared to controls. Syp expression was also distributed in a more generalized and confluent pattern in the SKA group than the RKA group. (SKA: one episode of NCSE; RKA: two episodes of NCSE; CTR: sham manipulated controls, Syp: synaptophysin I).

CHAPTER V

DISCUSSION

A substantial amount of knowledge on the now established detrimental consequences of CSE was obtained from animal models and preceded large epidemiological clinical studies, some of which are still ongoing (196). On the other hand, both clinical and preclinical studies on NCSE are still limited, and an electroclinically valid model for this condition is not well established. Here, we provided the first steps towards establishing a rodent model of NCSE to enhance our understanding of NCSE of temporal origin, and pave the way for more face validity-based preclinical research. Indeed, our experimental rats with KA-induced NCSE exhibited an electrographic pattern reminiscent of the EEG patterns of patients with temporal NCSE. At the same time, they manifested behavioral changes that mirrored the signs seen in humans with this condition, and these included behavioral arrest, head nodding, staring, and oromotor automatisms. In addition to its face validity, it is important to note the reliability of the herein described model, as it was reproducible with a high success rate in up to 90% of our experimental rats that experienced 20 to 130 minutes of NCSE with the above described electroclinical characteristics. In addition to establishing a successful model, we provided preliminary highly clinically-relevant data for potential harmful effects of two episodes of NCSE on hippocampal plasticity, and on cognitive and emotional behaviors.

In our behavioral studies, two episodes of NCSE resulted in detrimental consequences on cognitive abilities as reflected in the MAAV test, and lead to deficits in the FST and in the LDT that recapitulate some of the psychiatric comorbidities of the

epilepsies (depression and anxiety). MAAV, a test of emotionally-relevant learning, was developed in our laboratory to simultaneously assess the recognition of contextual and auditory cues, and the acquisition of adaptive electrical foot-shock avoidance behaviors that replace innate freezing behaviors. In this test, rats that underwent two episodes of NCSE exhibited a plateauing in the rate of learning acquisition of both context-cued and tone-signaled shock avoidance, hinting to a possible functional disruption of the amygdalohippocampal circuitry. In addition, while freezing was substantially replaced by avoidance behavior, rats that underwent two episodes of NCSE maladaptively maintained a higher level of freezing compared to controls in the retention subtest of the MAAV. Also in this preliminary data set, two episodes of NCSE resulted in increased depressive-like behaviors in the forced swim test, which is designed to examine learned despair and struggling behaviors. In addition, there was an NCSE-related decrease in the inter-chamber transitions in the light-dark box. In the absence of hypoactivity in the OFT, this decreased level of inter-chamber transitions was interpreted as a diminished exploratory tendency, and thus likely an increase in anxiety-like behaviors based on the historical drug-based predictive validity of the LDT. It is noteworthy that the employed behavioral tests are well established in our laboratory, and are highly objective and automated in terms of data acquisition and analyses. They are also tailored to models with prominent amygdalohippocampal circuitry involvement. Indeed, behavioral assessments such as conditioning tests, forced swim tests, and light-dark box tests are the most extensively employed paradigms in the literature in rodent models of temporal lobe seizures. For instance, in models of convulsive seizures, including those induced with KA, these tests show a slue of CSE-related cognitive and emotional behavioral deficits similar to the ones

we are reporting following NCSE. Taken together, our behavioral data suggest that hippocampal NCSE leads to a potentially persistent dysfunction in the amygdalohippocampal circuitry, as the deficits were detected 1-7 days after the electroclinical offset of NCSE episodes. If these behavioral changes prove to be significant and persistent, they point to a potential permanent harmful effect following NCSE, usually considered relatively benign in humans so far in terms of long-term sequelae. Indeed, NCSE is still treated less urgently when compared to CSE. The herein observed worsening in cognition and emotional behaviors have been described in association with a significant amount of neuronal loss in previous literature (156-158). However, in this work, there was no detectable overt hippocampal damage that could explain the observed behavioral worsening as assessed by structural staining, and immunostaining for reactive astrogliosis. On the other hand, interestingly, there were molecular changes that hint to a maladaptive neuronal activation and a consequently altered synaptic transmission, resulting in the formation of aberrant, potentially consolidated networks. Indeed, there was a seizure-induced maladaptive increase in Arc and Syp protein levels. Since Arc and Syp are known markers of, and likely contributors to hippocampal plasticity and learning (98,100,101,197-199), the herein observed changes in these proteins suggest that they play a role in NCSE-induced behavioral derangements. Our observed NCSE-induced behavioral disruptions, and their potential molecular synaptic underpinnings, point to a harmful effect of NCSE on hippocampal neuronal networks and an ensuing disruption in behavior if not promptly treated.

Intriguingly, one episode of NCSE resulted in improved cognitive behaviors, which is counter-intuitive, whereby a "brain insult" results in improving a survival-relevant learning task (avoiding electrical foot-shocks and a decrease in maladaptive freezing). Multiple landmark studies have shown that an insult to the hippocampus, including lesional ones, and KA-induced ones, can lead to enhanced performance in specific experimental behavioral paradigms, like standard active avoidance, where shocks are only signaled with a tone (200-202). Nevertheless, these published intriguing findings, along with our counter-intuitive data on a single NCSE-related enhancements in the MAAV test, are in frank contrast with both clinical and preclinical observations on hippocampal insults and lesions in TLE. Indeed patients with epilepsies of temporal origin, specifically those with hippocampal sclerosis, suffer from a multitude of cognitive disturbances (203-206). Along those same lines, experimental data on TLE, shows a detrimental effect of seizures on behavior and hippocampal neurons (207), in addition to altered neuronal signaling (208-210). A possible explanation of the herein observed counter-intuitive seizure-induced improved performance could be a hyperexcitability-dependent modulation in proteins involved in synaptic plasticity, learning and memory (80-83,104). Indeed, we observed an increase in the expression of Arc and Syp throughout the hippocampus of rats with two episodes of NCSE, and this increase was even higher and more confluently distributed in the hippocampus than both control and SKA rats. The fact that both RKA and SKA rats showed higher levels of Arc and Syp than controls with worse performance of the RKA group in the fear conditioning test brings forth the argument of a potential delicate molecular balance in synaptic machinery proteins for optimal neuronal functioning. Indeed, findings similar to our work are not uncommon, as they have been described in studies

depicting a delicate balance in physiological Arc expression and translation mechanisms, the disruption of which may contribute to certain neurological conditions including autism spectrum disorder (211-213).

In this work, the seizure-induced maladaptive increases in Arc and Syp was associated with behavioral disturbances in the RKA group, but a higher increase in these proteins was associated with improved behaviors. This evolutionary counter-intuitive hyperexcitability-induced hippocampal behavioral facilitation findings in our work and in prior literature can be explained by either (a) an early effect that will be followed by chronic worsening due to an NCSE-induced aberrant synaptic reorganization, or (b) a beneficial effect in one the MAAV panel but a net loss of temporal lobe functions when other panels are tested. The observed changes in marker of synaptic plasticity, Arc and Syp support the former hypothesis, whereby a temporary deceptive enhancement in cognitive performance and synaptic plasticity precede an eventual worsening in neural functioning and in behavior. Indeed in addition to the observed circumstantial association between their levels and the relatively early NCSE-induced behavioral changes, the contribution of Arc and Syp alterations to these changes is also biologically plausible based on some established properties as described below.

Arc is so far one of the very few immediate early genes in which both RNA and protein products are localized to dendrites (83,214,215), and more importantly it is regulated by synaptic activity (214). Arc expression is also induced by long-term potentiation mechanisms (98,216), in addition to physiological neural activity (214,217), whereby LTP-induced local Arc translation could be followed by dendrite-wide changes in

homeostatic plasticity and enhanced synaptic strength (216). Recent evidence also suggests differential activity-dependent regulation of Arc RNA synthesis, localization, translation, and metabolism (216).These properties qualify Arc as a unique immediate early gene among its prototypical counterparts such as zif-268 and c-fos, as it undergoes many levels of regulation from gene expression to trafficking and local regulation and translation. In this work, Arc protein levels were increased in specific hippocampal areas tightly related to KAR and AMPAR predominance in the CA3/CA4 and dentate gyrus area (86,90,218-220). This observed increase points to potential efficient recruitment of synaptic networks likely involving AMPAR trafficking and lowered LTP thresholds (80-83,92-95,104). Similarly, though not as extensively studied as Arc, Syp, a protein involved in synaptic transmission had higher levels of expression in rats treated with one episode of NCSE. Those findings suggest that there might be a correlation between neuronal activation and reactive synaptic plasticity mediated by Arc and Syp respectively. The fact that prior neuronal activation and the resulting changes in synaptic plasticity are associated with improved later learning has been explained with emerging newly described mechanisms that have been referred to as a synaptic tagging and neuronal allocation (221). Along those same lines, due to their electrophysiological and molecular properties amenable to rapid alterations, the hyperexcitability-induced increases in Syp and Arc, could be contributing to mechanisms similar to synaptic tagging. This suggested phenomenon requires further exploration, making it the subject of ongoing investigations in our laboratory.

In summary, NCSE, whether in one or two episodes, elicited potentially permanent perturbations in synaptic plasticity, and it more importantly lead to detrimental behavioral

changes. This will prompt us to engage in further laboratory investigations, approaching this condition more meticulously in terms of transient and potentially permanent preclinical and clinical behavioral, emotional and molecular sequelae. Indeed, on one hand, our molecular behavioral and morphological data point to the clinically relevant finding of NCSE-related early harmful changes in plasticity and function, which may be potentially permanent. On the other hand, our data also point to an intriguing scientific finding that can be possibly explained by an early potentially transient synaptic enhancement, and this may also explain a literature on counter-intuitive brain insult-related hippocampal facilitation and improved learning.

CHAPTER VI

LIMITATIONS AND FUTURE PERSPECTIVES

This work sheds light on potentially threatening consequences of NCSE on brain electrophysiology, cognition and emotional behaviors. The study however, has some limitations that can be traced back to time restraints, given the behavioral nature of many of the outcomes in this work. Indeed, behavioral experiments in general require a larger number of rodents in order to be sufficiently powered in order to establish statistical significance, which was not possible to achieve within the timeframe allocated to this work. In addition, Arc and Syp protein levels are not yet fully assessed with densitometric quantifications. Finally, there is a role for longer EEG recordings during the period of behavioral testing in order to confirm the lack of seizure recurrence and thus their potential contribution to the herein reported behavioral disturbances.

More research is still needed to dissect the sequelae of NCSE and its exact mechanisms. This has clinical implications on gauging treatment urgency, and tailoring the index of suspicion, especially in those who are susceptible to NCSE, such as TLE patients, especially in the presence of prominent and not fully explained cognitive and psychiatric comorbidities. Future studies will focus on the completion of the data set in terms of acute and long-term effects of one or two episodes of NCSE, as well as on the mechanisms of the herein obtained counter-intuitive NCSE-induced improvement in learning.

CHAPTER VII

CONCLUSIONS

Recurrent episodes of NCSE could potentially bear permanent detrimental consequences on cognitive and emotional behaviors. The NCSE-related synaptic changes in one or two episodes alarmingly hint to an aberrant synaptic plasticity that will probably have chronic effects on the electrophysiological homeostasis of the hippocampus and its functions. Consequently, NCSE is shown to likely be a much more serious condition than is generally appreciated. Indeed, in current practice, it remains underdiagnosed, not promptly treated, and lacking universal treatment protocols. Moreover, it is a highly heterogeneous clinical condition that is understudied in the pediatric population, especially that its manifestations can be as subtle as inexplicable changes in behavior, language and memory capacities, and school performance. Based on the herein reported data, a more prompt recognition and more emergent treatment than is currently being done may be necessary in clinical practice to prevent potential NCSE-related brain damage. Ongoing work in our laboratory aims at confirming our highly clinically relevant data.

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