

AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF NON-CONVULSIVE SEIZURE BURDEN  
ON COGNITION AND EMOTIONAL BEHAVIOR IN PERI-  
ADOLESCENT RATS

by  
NOUR EL AYOUBI

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for the degree of Master of Science  
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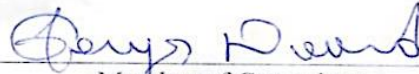
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# ABSTRACT OF THE THESIS OF

Nour El Ayoubi

for

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Title: The Effect of Non-Convulsive Seizure Burden on Cognition and Emotional Behavior in Peri-adolescent Rats.

**Background:** Non-Convulsive status epilepticus (NCSE) consists of prolonged seizures without tonic muscle stiffening or clonic rhythmic activity. In a clinical setting, NCSE is often underdiagnosed due to difficulties in recognizing its manifestations (altered consciousness, blanking, automatisms), and therefore it frequently recurs prior to coming to medical attention. Moreover, because the potential harmful effects of NCSE on the immature brain remain elusive, the urgency of its diagnosis and aggressivity of its treatment are controversial. Previous work in our laboratory revealed that NCSE in peri-adolescent rats is associated with an early hippocampal synaptic dysfunction and ensuing learning deficits.

**Aim:** We aim at investigating whether these short-term harmful effects persist later in life leading to neurodevelopmental consequences and whether they are reinforced by recurrence.

**Methodology:** One or two episodes of NCSE were induced via stereotaxically-delivered intrahippocampal kainic acid (KA) in peri-adolescent rats at post-natal day 42 (P42) under EEG monitoring (The LSK group received one KA injection while the LRK group received two injections. The LCTR group received saline injections). We performed behavioral tests (P72) for auditory and contextual learning (modified two-way active avoidance test), and visuospatial navigation and memory deficits (Morris water maze test). We also assessed emotional behaviors such as hyperactivity, exploratory and anxiety-like behaviors through open field and light dark tests, and depressive-like behaviors through the forced swim test. Continuous EEG monitoring was done for a duration of one month before behavioral testing and one week post behavioral tests. Potential hippocampal damage and synaptic dysfunction were assessed histologically via immunohistochemistry (neuronal counts and synaptophysin, respectively).

**Results:** All induced seizures were of almost equal duration and latency between the rats. No seizure recurrence was detected. During the probe trial in the Morris water maze test, and as compared to the control group, the injured groups of rats spent less time in the probe quadrant, which shows visuospatial, memory, and retention deficits post NCSE.

**Conclusion:** The preliminary data shows that NCSE causes visuospatial navigation and memory deficits. Knowing these harmful effects proves that further research should be done to start diagnosing and treating NCSE more timely and aggressively.

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## ABBREVIATIONS

AMPA  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

CSE Convulsive Status Epilepticus

EEG Electroencephalography

FST Forced Swim test

GABA Gamma-Aminobutyric acid

GFAP Glial Fibrillary Acidic Protein

KA Kainic Acid

KAR Kainate receptor

LCTR long-term control

LDT Light-Dark Box Test

LRK long-term repeated kainic acid

LSK long-term repeated single kainic acid

MAAV Modified Active Avoidance

MWM Morris Water Maze

NCSE Non-Convulsive Status Epilepticus

NE North East

NMDA N-Methyl-D-aspartic acid

NW North East

OFT Open Field Test

P Postnatal day

PFA Paraformaldehyde

SE Status Epilepticus

SW South West

Syp Synaptophysin

TLE Temporal Lobe Epilepsy

# CHAPTER I

## INTRODUCTION

The brain and the nervous system are the most complex structures of our human body. Until this date, the human brain and its neuronal networks remain the most perplexing discoveries. However, thanks to the advancement of scientific research, scientists have been able to reveal many of the brain's mysteries. Like all organ systems in the human body, the brain and its neuronal networking system must be maintained within the physiological homeostasis for proper functioning.

An epileptic seizure is a result of disrupted balance in the nervous system. It consists of abnormal excessive, or synchronous neuronal discharges, which are manifested clinically with sudden motor, sensory, or behavioral changes with or without various levels of alterations in consciousness (1). It is caused by a disruption to the brain cells and an interruption to their electrical impulses. Seizures can be provoked or unprovoked. A seizure can be provoked by an acute systemic illness or brain insult (trauma, infection, toxic exposure, or high fever); or unprovoked as a result of a genetic or acquired epileptogenic network (2). Two or more unprovoked seizures define epilepsy, which is a neurological disorder that involves abnormal brain cell activity, and that leads to neurological, cognitive, behavior, and psychological detrimental effects (2). Seizures can resemble tonic-clonic convulsions or they can resemble non-convulsive manifestations only.

### **A. Status Epilepticus with a Focus on Non-Convulsive Status Epilepticus**

Status epilepticus is seen in patients with prolonged seizure activity. It is caused by the failure of seizure termination and results in severe neuronal damage. In

epilepsy, excitatory and inhibitory mechanisms are altered. During a seizure, inhibitory GABA receptors decrease, while excitatory NMDARs increase, due to increased synaptic trafficking in the neurons (3,4). Thus, hyperexcitability occurs which results in prolonged seizures. Statistics show that patients who exhibit one episode of prolonged seizures have almost 10-30% of seizure recurrence during their lifetime and a 20-40% risk of having epilepsy (5,6). Patients show different symptoms of seizures which is why status epilepticus is divided into two types: Convulsive status epilepticus (CSE) and non-convulsive epilepticus (NCSE).

Non-convulsive status epilepticus (NCSE) consists of prolonged seizures without tonic muscle stiffening or clonic rhythmic activity. (7,8).

However, there is a lack for a clear definition of non-convulsive status epilepticus, which urges more research on the topic. 20% -40% of patients with status epilepticus lack convulsive events and are of the non-convulsive type. (8). In addition, some suggested that it accounts for up to 20% of all cases of prolonged seizures in hospitals and up to 47% in the intensive care unit (9). Yet, these statistics are often contradictory, due to frequent misdiagnosis. In a clinical setting, NCSE is often underdiagnosed due to difficulties in readily recognizing its manifestations, and therefore, it frequently recurs prior to coming to medical attention (10). Clinical manifestations include cognitive impairment, speech arrest, subtle facial, trunk, or limb twitches, head, automatisms, and abnormal behaviors including blanking, starring, and hallucinations (11,12). These manifestations may challenge the diagnosis, as such symptoms can arise from many factors. Also, we still lack an agreed time point after which these seizures cause damage and after which

immediate intervention is needed. In fact, the treatment strategies of NCSE remain debatable.

## **B. Epilepsy**

Seizures are abnormal excessive neuronal firing that lead to abnormal behaviors (13). A seizure results from the malfunction of a net balance between inhibitory and excitatory receptors that mainly include GABAA (gamma-amino-butyric acid) ionotropic receptors, NMDA receptors, AMPA receptors, and kainate receptors (KARs) (14). Malfunction of these receptors disrupts the homeostasis of the brain which causes a shift in the threshold of neuronal excitability; thus, hyperexcitability leads to seizures. This disruption is measured by an electroencephalography (EEG). EEG supports diagnosis by revealing areas of hyperexcitability reflected by spikes and sharp waves. A seizure on an EEG is characterized as fast electrical waves with an evolving abnormal rhythm that overrides the brain's normal rhythm.

The seizure circuitry involves the following: 1) Seizure focus that is the neuronal region where seizure starts, 2) initiation circuits that are nearby neuronal circuits that initiate seizure sustainability, 3) secondary pathways that spread the seizure to further neuronal regions which leads to generalized seizures, and 4) modulatory regions that are different neuronal circuits that modulate the seizure severity which modulates the chance of occurrence (15). Based on this, seizures are categorized into generalized seizures, focal seizures, or unknown originating seizures. Focal seizures start from one hemisphere (and may spread to the other hemisphere causing generalized seizures) while generalized seizures begin in both hemispheres bilaterally. Symptoms in each clinical case differ between different seizures types and the onset origin.

Temporal lobe epilepsy TLE is a type of epilepsy that involves focal seizures that are resistant to treatments. TLE appears in the mesial temporal lobe, namely the hippocampus, amygdala, and parahippocampal gyrus (16). TLE is accompanied by several psychological and cognitive problems. TLE patients demonstrated poor performance on concentration and social recognition in addition to anxiety and depression (17). In rat models, rats with TLE demonstrated poor performance on recognition tasks and spatial memory (18).

### **C. Amygdalo-hippocampal function and circuitry**

#### ***1. The hippocampus***

The hippocampus is one of the most vital parts of the limbic system. Human and non-human primates have two hippocampi (one in each hemisphere) that are located in the anterior part of the medial temporal lobe. Each hippocampus is divided into cornu ammonis subfields CA1-CA3, the dentate gyrus, and the subiculum, in addition to more extended regions that are the entorhinal, perirhinal, and parahippocampal cortices. (19). The dentate gyrus contains the CA4 region that is known as the hilar region. The cornu ammonis subfields consist of pyramidal neurons while the dentate gyrus consists of granule cells. These two regions are connected to each other and to other brain regions by the Papez circuit (20). Most of the neuronal input to the hippocampus is received by the perforant pathway that starts from the entorhinal cortex reaching the dentate gyrus. The CA1 subfield sends excitatory axons to the subiculum and the entorhinal cortex (21). The dentate granule cells send projections (mossy fibers) to the hilar region and CA3 pyramidal cells, along with dendrites that extend to the dentate molecular layer.

The hippocampus involves many crucial functions that include memory, emotions, behavioral cognition, decision making, and spatial navigation (22). The hippocampus is

a polymodal association area that transforms sensory information to complex configurational neural representations (21). Different regions and developmental stages of the hippocampus are function specific (19,23). The ventral hippocampus in rats, that designates to the anterior part of the human hippocampus, is mainly in control of stress and emotions; while the dorsal hippocampus in rats, that designates to the posterior part of the human hippocampus, is mainly in control of spatial navigation and cognitive functions (24,25). Studies show that the hippocampus is also involved in pattern differentiation and in “fight or flight” anxiety situations (26,27). In order for these functions to be executed, the hippocampus coordinates with several other structures of the brain, like the amygdala.

## ***2. The amygdala***

The human brain has 2 almond shaped nuclei as part of the limbic system, each called the amygdala, that are located in the medial temporal lobes in front of the hippocampus (21,28). The amygdala consists of dozens of nuclei and shares reciprocal connections with the hippocampus and many of its extended regions (29). The amygdala consists of 3 main group of nuclei that are: The cortical amygdalar nuclei, the basolateral amygdalar nuclear complex, and the the centromedial nuclei group (30).

Studies show that the amygdala plays a key role in emotions and learning, particularly in processing fear and reward stimuli. In addition, many studies show that it is involved in emotional learning and memory by modulating hippocampal synaptic plasticity (31). The amygdala also gives an emotional sense to basic sensory stimuli by receiving projections from sensory association areas and the thalamus, and by

integrating this information. (21,32). The basolateral nuclear complex of the amygdala is responsible for fear conditioning which is a type of emotional learning (33).

### ***3. Disruption of the amygdalo-hippocampal circuitry and TLE***

Any dysregulation in the amygdalo-hippocampal circuitry results in neurological deficits including emotional and cognitive deficits. For example, Alzheimer's disease is one of these deficits which results from the accumulation of neurofibrillary tangles and amyloid plaques in the temporal lobe, including the hippocampus and amygdala (34). Another important example is TLE where the removal of the temporal lobe with the hippocampus and amygdala relieves 80% of patients from seizures (31).

This is why emotional and cognitive deficits are seen in TLE patients, in addition to spatial memory deficits are seen in TLE patients (35,36). Damage of the dorsal hippocampus which it projects contextual information to the amygdala for fear conditioning and processing may lead to loss of cued fear conditioning by context stimuli might also be reported in these patients in case of (37) Damage to the amygdala is also involved in psychological disorders such as depression and aggressive behavior in epileptic patients (38).

### ***4. Behavioral tests used to assess the function of the amygdalo-hippocampal circuit:***

Many behavioral tests have been used to test for the functionality of the hippocampus and amygdala. Through these tests, scientists have been able to assess cognitive and emotional functions in humans such as depression, anxiety, memory, and learning (39). These tests were used to explore deficits in induced status epilepticus (SE) and were then translated to a clinical setting. Some of these tests include: The

light-dark test which is used to assess anxiety-like behaviors, the open field test which is used to assess anxiety-like and exploratory behaviors, the forced swim test which is used to assess depressive-like behaviors, the Morris water maze test which is used to assess visuospatial navigation and memory, and the Modified Active Avoidance test which is used to assess the learning and memory of auditory and contextual emotional cues. The MAAV test is an expanded version of the Pavlovian conditioning test (37). Animal's behaviors are observed in these tests and are used to measure emotions (Table 2).

Table 1-COMMON BEHAVIORAL TESTS USED

Behavioral test	Used to assess	Measurements
Light dark test (LDT)	Anxiety-like behaviors	Time spent in each chamber, number of entries to each chamber
Open field test (OFT)	Anxiety-like and exploratory behaviors	Distance traveled, time spent in each zone, time spent exploring central objects
Forced swim test (FST)	Depressive-like behaviors	Percentage immobility and struggling during swimming
Morris water maze (MWM) test	Visuospatial navigation learning and retention	Using visual cues to reach an escape platform

Modified active avoidance test (MAAV)	Emotional-related learning	Percentage of shock avoidance and time needed to avoid the shock
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#### **D. Animal models used in the epilepsy research**

Most animal models of epilepsies are very controversial. It is good to note that there is no “perfect” animal model for the study of NCSE. However, established models induce NCSE in the areas involving the limbic system (specifically the hippocampus, amygdala, and entorhinal cortex), as these areas involve the lowest threshold for evoking seizures (40). Also, it was found that the induction of NCSE in these brain areas manifests in animal behaviors in a way that is similar to human seizure manifestations (41). Detecting these manifestations in animals can be limited and wrongly interpreted, which is why the report of these manifestations should always be correlated with EEG recordings. Studying cognitive aspects of animals can be challenging because it is much less complex than in humans, and this is a limitation of using animal models in epileptic research. Most often, male rodents are preferred over female rodents since hormonal factors (like estradiol) affect sensitivity to chemo-convulsants used in the induction of seizures (42). In fact, it was suggested that testosterone increases the susceptibility to seizures. (42). Chemo-convulsants can be administered systematically or focally. However, focal administration is always preferred since it decreases mortality rates, and uses much less pharmacological agents, thus rendering it less expensive. Also, focal administration minimizes the possible side effects of the chemo-convulsant. The Racine scoring system is used to evaluate seizure

severity based on animal behavior and manifestations. It was first described 1972 and updated based on new animal models (43). The Racine scoring system resembles five stages which are: 1- Mouth and facial movements, 2- Head nodding, 3- Forelimb clonus., 4- Rearing, 5- Rearing and falling (44). These stages further classify limbic status epilepticus into 4 types: Type I (which is known as immobile status) which is characterized by blanking and motionless starring, type II (known as exploratory status) is characterized by discontinuous and meaningless exploratory behavior by the experimental animal, type III (known as masticatory status) is characterized by repeated facial and forelimb convulsions and automatisms, and type IV (known as clonic status or generalized status) is characterized by generalized clonic or tonic activity (43) (see Table 2).

Table 2 RACINE SCALE-Table showing classification of SE in animal models based on the Racine scale

<b>Stage Based on Racine Scale</b>	<b>Name</b>	<b>Characteristics</b>
<b>1</b>	Immomible Status	Blanking and motionless starring
<b>1,2</b>	Exploratory Status	Discontinuous and meaningless exploratory behaviors
<b>3</b>	Masticatory Status	Repeated facial and forelimb convulsions and automatisms

4,5	Clonic or Generalized Status	Generalized clonic or tonic activity
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### 1. Kainic acid (KA) animal model of status epilepticus

Kainic acid, which is a synthetic cyclic analog of L-glutamate, is a powerful excitotoxic agent that causes prolonged seizures when injected. It binds to special receptors called kainite receptors, which are an ionotropic glutamate receptor type (45). These receptors are especially found in the ventral thalamus, hippocampus, striatum and olfactory bulb of the rat forebrain (45). They are also involved in the modulation of inhibitory and excitatory neurotransmitters release and the synaptic network activity regulation (46). The activation of these receptors causes seizures arising from the limbic structures of the brain (46). It is suggested that seizures resulting from systematic administration of KA are reported to start in the entorhinal cortex and spread via the rostral Ammon's horn, the amygdala, the medial thalamus and the pre-frontal cortex into the hippocampus (46). However, it was reported that the rodents receiving KA intracerebrally are calmer than those receiving KA systematically (47). Recently, it was found that an intrahippocampal low dose of KA injected unilaterally induces neurotoxic NCSE event (48,49). These direct neurotoxic effects are mostly observed (when KA is delivered into the hippocampus) on the pyramidal neurons of CA1 and CA3 regions of the hippocampus, the dentate hilus, and even outside (47). Another study reported the increased expression of kainate receptor subunits in reactive hippocampal astrocytes and its surrounding cortex after induced status epilepticus, which may be a possible contributor to the epileptogenic process and the rise of spontaneous seizures (50). In such a situation, KA is usually divided into smaller doses that are given gradually to

minimize mortality rates (51). Limitations of this model include a disruption of blood-brain barrier and a possible brain injury by cannula placement surgery for the injection of KA.

## **2. Pilocarpine animal model of status epilepticus**

The pilocarpine model was first described in the 1980s, and is characterized by a dose of 100–400 mg/kg administered intraperitoneally in rodents (52). Pilocarpine is a muscarinic acetylcholine agonist that evokes excitatory activity when it binds to its M1 acetylcholine receptor of the muscarinic type (53). Pilocarpine can be administered by intraperitoneal or intrahippocampal injections (54). It was shown that pilocarpine induced seizures start in the ventral forebrain, where the nucleus accumbens is possibly the most severe injured area due to high presence of muscarinic receptors in this area (55). Limitations of this model include high mortality rates and high toxicity levels. Attempts of decreasing the dosages or splitting them was time consuming and failed because this led to high threshold seizure induction, in addition to that it is time consuming (56). To deal with this issue, the lithium-pilocarpine model has been established (57). This model is characterized by administering lithium as a pre-treatment before the administration of pilocarpine (51). Another limitation is that this model is associated with prolonged and diazepam insensitive seizures (58).

## **3. The kindling animal model of status epilepticus**

The kindling model is characterized by repeated electrical stimulations in the brain via implanted electrodes (51). This is a well-known model where it produces less neurological damage than the chemoconvulsant model (KA and pilocarpine) (58).

However, limitations of this model include costs, skills, and time. It requires skills from the researcher's end to avoid damaging the implanted electrodes that are costly (51).

Another limitation is that this model has a less spontaneous recurrent seizure incidence, and a lack of seizure contribution to neuronal damage, which is not comparable to what is seen with temporal lobe epilepsy (59).

Before establishing an animal model of status epilepticus, a researcher must consider several factors like the costs, time, efforts, materials, and mortality rate of the animal model.

#### **E. Current studies done on NCSE**

Studies reporting the cellular and structural damage to the human brain post NCSE are still absent. However, very few pre-clinical studies reported the potential damage and consequences of NCSE. It has been reported that NCSE leads to gradual brain pathology in rodents post 1,2, and 4 weeks of NCSE. In addition, it has been reported that NCSE causes long-term social behavioral deficits in rodents in addition to motor deficits. However, despite the damage reported, another study that induced NCSE in guinea pigs showed the absence of cell loss contralateral to the seizure induction site.

Some alarming studies reported the consequences of NCSE on cognition and learning. Interictal epileptic spike patterns are usually seen in status epilepticus and it was seen preceding a seizure activity. These interictal epileptic spikes were associated with decreased IQ performance and language proficiency in children with focal epilepsies (66-70). Another study found deficits in alertness, attention and short-term memory function following non-convulsive seizures (71).

## CHAPTER II

### AIMS AND HYPOTHESIS

While convulsive status epilepticus is studied using well-established animal models and clinical cases, there is a lack in studies of NCSE and its potential harmful effects.

Moreover, since the consequences of NCSE on the brain are still not well studied and known, the protocol for its diagnosis and treatment is controversial. This calls for urgent further research on this condition. Previous work in our laboratory revealed early detrimental effects post NCSE. On the behavioral and molecular level, our work showed that NCSE leads to early learning deficits accompanied by hippocampal synaptic dysfunction (62). Here, we investigate whether these short-term harmful effects persist later in life.

**Aim 1:** To confirm whether the early learning deficits (emotional related learning and visuospatial navigation memory and learning) persist in the late life phase, one-month post NCSE.

**Hypothesis 1:** KA induced hippocampal NCSE negatively impacts the function of the amygdalo-hippocampal circuitry leading to cognitive and learning deficits.

**Aim 2:** To confirm whether one or two episodes of induced NCSE cause long-term emotional behavior deficits, such as anxiety and depression, and whether these deficits are affected by the seizure burden.

**Hypothesis 2:** One or two episodes of NCSE lead to long term seizure-burden dependent emotional behavior deficits.

Aim 3: To investigate the late life molecular damage post NCSE, by evaluating hippocampal neuronal cell count, glial fibrillary acidic protein (GFAP) expression, and synaptophysin (Syp) protein levels, the synaptic plasticity marker.

Hypothesis 3: One or two episodes of NCSE will elicit changes in Syp levels and GFAP expression, that persists in later life stages and is exacerbated by seizure burden.

# CHAPTER III

## MATERIALS AND METHODOLOGY

### **A. Animals and experimental design**

In this study, male Sprague Dawley rats, housed at an optimal constant temperature (25 °C) and a 12-hour light dark cycle with unlimited access to food and water, were used. All procedures performed were approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut (AUB) and limiting rat's discomfort during the experimental procedure was ensured.

A long-term paradigm was implemented in this study with a total number of 35 rats. Intra cannular hippocampal surgeries were done at post-natal day (P35) after which rats rested for several days before NCSE induction. One or two episodes of NCSE were induced at P42-43 upon injecting intra-hippocampal kainic acid (KA) under continuous EEG monitoring for one month. NCSE was evaluated based on EEG activity and behavior (according to Racine's Scale). Then, behavioral testing was performed at P72 and included: Light-dark test (LDT), open field test (OFT), forced swim test (FST), Morris water maze test (MWM), and modified active avoidance test (MAAV). Finally, rats' brains were perfused with Paraformaldehyde (PFA) and collected for histological analysis. Some rats were sacrificed at the last day of testing (P93), 45 min post MAAV for SYP (synaptophysin) staining, while other rats underwent EEG recording for 1-week post-behavior and were sacrificed at P105 for NeuN (neuronal count) staining. (See figure 1)

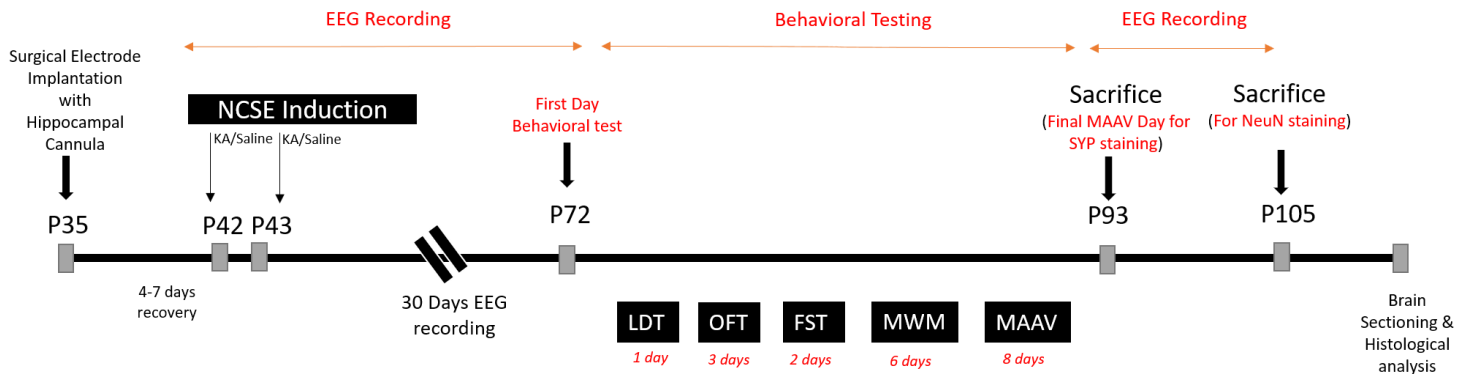
The rats were divided into groups as follows:

**LRK group:** Two episodes of NCSE were induced by two 24-hours apart intrahippocampal KA injections, 0.00625  $\mu\text{g}$  in 0.2  $\mu\text{L}$  saline each, and dissolved in normal saline. This is a sub convulsive dose which results in an electrical brain activity and behavioral manifestations comparable to those seen with NCSE.

**LSK group:** One episode of NCSE was induced by an intra-hippocampal injection of KA (0.00625  $\mu\text{g}$  in 0.2  $\mu\text{L}$  saline), after which the rats received an intrahippocampal injection of normal saline on the second day of induction.

**LCTR group (control group):** Sham manipulation was performed by injecting two 24-hours apart intrahippocampal doses of normal saline (the vehicle) to account for volume induced mechanical excitation, if any.

Rats that failed to show seizure activity after KA administration were excluded from the study.

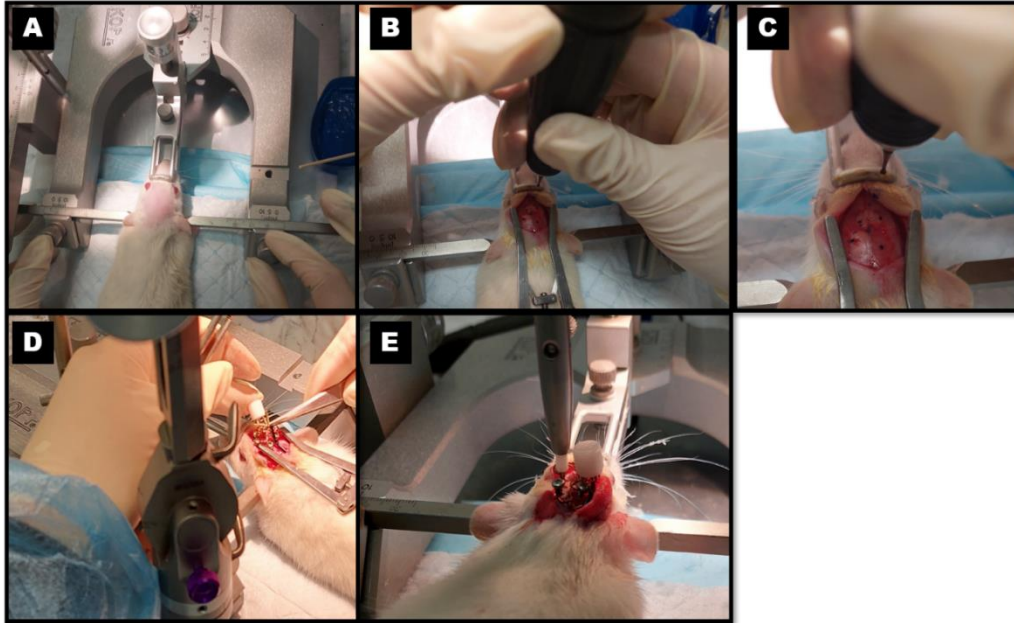


**Figure 1 Experimental paradigm.** After Surgical Electrode Implantation with Hippocampal Cannula at P35, rats rested for 4-7 days and were then either injected with KA for NCSE induction or with saline. After one month of continuous EEG recording, rats underwent several behavioral tests starting at P72. Finally, rats were sacrificed for histological analysis.

## **B. Stereotaxic Implantation of Electrodes with an Intra-hippocampal Cannula**

Implantation of epidural electrodes and cannula was done for efficient EEG recording on several rats collectively. First, anesthesia (mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg)) was administered intraperitoneally and maintained throughout surgery. After ensuring that the rat was properly anesthetized (by slightly pinching the feet to check for signs of pain), its head was properly shaved from between the eyes until the back of the ears using a trimmer and then was tightly secured on the stereotaxic frame (Figure 2-A). The scalp was sterilized by iodine followed by ethanol, and then, a 2 cm midline incision was made by a sterilized blade exposing the skull using the retractor (Figure 2-B). After that, the soft tissue was scraped and cauterized using 3% hydrogen peroxide. A high-speed drill was used to carefully drill small 1.4 mm holes in the skull in order to place the epidural electrodes (Figure 2-C). Five electrodes were tightly screwed to the drilled holes which included: Left and right frontal electrodes (F3 and F4: 2 mm anterior to, and 3 mm lateral to the bregma respectively), left and right parietal (P3 and P4: 5 mm posterior to, and 3 mm lateral to the bregma respectively), and one anterior midline reference electrode (Ref: 6 mm anterior to the bregma) based on the Paxinos and Watson adult rat brain atlas. A ground wire was also placed under the skin at the base of the neck. As for the intrahippocampal cannula (2.6 mm in length), it was implanted stereotaxically onto the CA1 region of the left hippocampus (2.4 mm posterior, and 2 mm lateral to the bregma) (Figure 2-E). All the sockets extending from the electrodes and the ground wire were inserted in a sixth channel pedestal that was secured by acrylic dental cement (Figure 2-D). All rats were transferred to single animal cages individually and left to rest for 4-7 days with proper

observation before seizure induction. Postoperative pain management was done by the administration of paracetamol (1mg/ml) for 3 consecutive days



**Figure 2 Stereotaxic Implantation of Electrodes with an Intra-hippocampal Cannula. Panel A.** The head was shaved and tightly secured on the stereotaxic frame. **Panel B.** The skull was exposed then properly sterilized and the bregma was located. **Panel C.** Using a high-speed drill, 5 small 1.4 mm holes were made in the skull for electrode placement. **Panel D.** All the sockets extending from the placed electrodes and the ground wire were inserted in a sixth channel pedestal. **Panel E.** Intrahippocampal cannula was implanted stereotaxically.

### C. EEG recording

Baseline brain EEG was recorded few hours before seizure induction for each rat in specialized plexiglass EEG cages where the pedestal was connected to a customized cable that accommodates the movement of the rats and which is connected to the EEG recording system (Xltek, Natus Medical, USA). The EEG recordings were read by two readers blinded to the treatment groups. During seizure induction, NCSE onset and offset, in addition to the duration and latency, were determined. Long term EEG recordings were also done for one month and reviewed (pre and post behavioral testing) to check for any seizure recurrence or abnormal brain electrical activity.

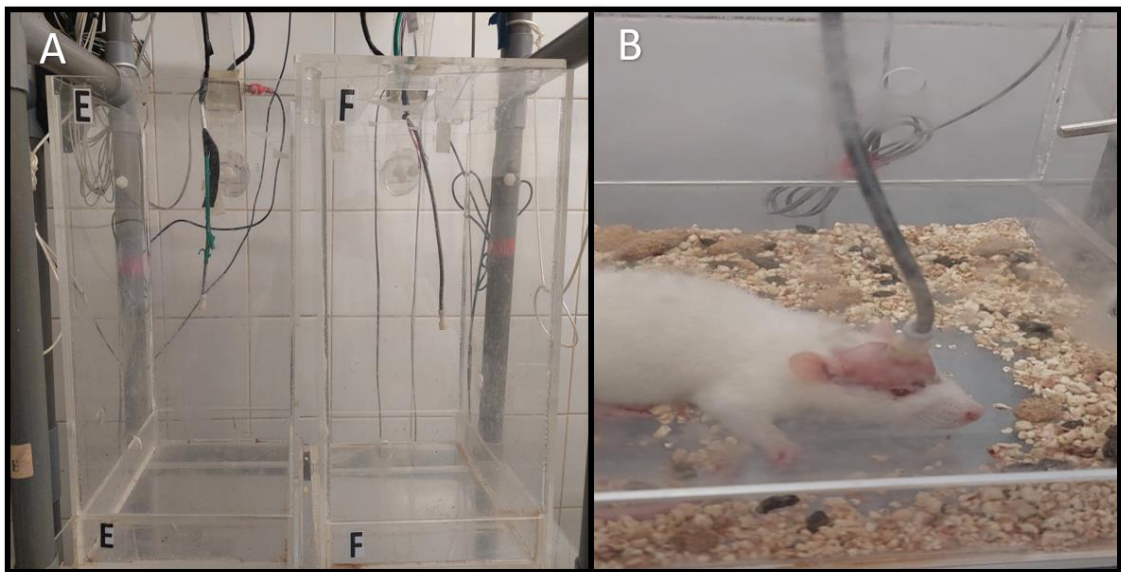


Figure 3 **EEG recording.** **Panel A.** Customized EEG cages with commutators for recording. **Panel B.** The pedestal was connected to the commutator that allows free movement for the rat without affecting the EEG recording.

### D. Behavioral tests

All rats were put through a full panel of behavioral tests at P72 in order to study any cognitive or emotional abnormal behaviors. These behavioral tests were performed in an order of least to the most aversive as the following: Light-dark test (LDT), open

field test (OFT), forced swim test (FST), Morris water maze test (MWM), and modified active avoidance test (MAAV).

### ***1. Light-dark test***

The light dark test was done to check for anxiety-like and exploratory behaviors. This test consists of a single 5 min session where the rat is put in a shuttling box (Coulbourn Instruments, Harvard apparatus, USA) that is divided into two equal compartments (H: 34 cm, W: 27 cm, L: 27 cm), linked by a  $9 \times 9$  cm door located in the middle of a metallic partition wall (Figure 4), and is allowed to freely explore the environment. The left chamber is lit with white foam panels on the walls, while the right chamber is dark and covered with black foam panels. The box was cleaned between the rats by an odorless detergent followed by 70% ethanol. The time spent in each compartment and the number of shuttling between compartments was obtained using Graphic State 4

software (Coulbourn Instruments, Harvard Apparatus, USA).



Figure 4 **Light dark box apparatus.** The right chamber is dark with no illumination and black walls while the left chamber is lit with bright illumination and white walls. Two cameras are placed above each box for video recording.

## ***2. Open field test***

The open field test was done to test for hyperactivity, exploratory, and anxiety-like behavior. Rats' movement was video recorded over 5-minute sessions for three consecutive days, in an opaque plexiglass square field (W 80 cm, L 80 cm, H 40 cm) (Figure 5). On each day, a different novel object was added in the field's center (starting with a cube, a ball, and finally a bottle). The rats were placed in the closest corner to the newly added object; and were then allowed to freely roam. The apparatus was cleaned between the different rats with odorless detergent followed by 70% ethanol solution. The rats' motion was then analyzed using the SMART video tracking 3.0 software (Panlab, Harvard apparatus, USA).

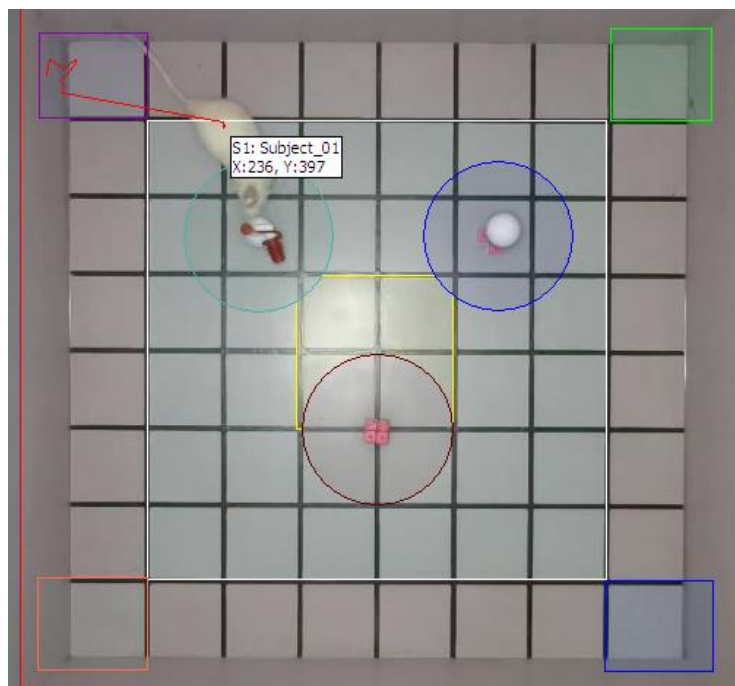


Figure 5 **Open field test**. The box is divided into multiple zones. As the rat moves freely inside the box and explores the objects placed in the center of the box, time and distance travelled in each zone is being calculated.

### ***3. Forced swim test***

This was performed to study depressive-like behaviors in rats. The test consists of 10-minute sessions for two consecutive days. In each session, rats were placed in a clear transparent plexiglass cylinder (Figure 6) (20 cm in diameter and 50 cm in height (Coulbourn Instruments, USA)), filled with water of temperature 25°C to a height of 35 cm. Rats were then left to swim over the conducted session and were video recorded. After each session, water was changed, and the rat was put in a cage with an absorbent towel under a heat lamp in order to dry up. Finally, rats' swimming behavior (percentage of time immobile) was analyzed using the SMART software where a detection activity threshold was fixed to account for the rats' movement. Immobility was defined

by no movement or minimal movement which is required to keep the body and head floating.



Figure 6 **Forced Swim test Setup**. Each cylinder was filled with water and the rats were left to swim for 10 minutes per session. Rats were video recorded while swimming using the camera shown in the figure.

#### **4. Morris water maze test (MWM)**

This test was performed to assess visuospatial navigation and memory. A dark blue plastic circular pool (150 cm in diameter and 80 cm in height (Coulbourn Instruments, USA) filled with water of 25°C temperature and 30 cm depth was used. The pool was virtually divided into four quadrants (north east, south east, north west, south west) by two imaginary intersecting perpendicular lines (Figure 7-A). Visual cues were hanged on the surrounding room's walls and kept constant throughout the test (Figure 7-B). Each rat was first habituated by being allowed to freely swim in the water-filled pool for 2 minutes. Learning

acquisition was then performed for 5 consecutive days by placing an invisible platform 2 cm below the water surface in the north east quadrant, and immersing the rat in the water facing the pool's wall on different points. For five days, each rat was allowed to freely swim and find the platform over four consecutive training trials with a 30 second rest in between. In case the rat fails to find the platform, it was placed on it for 30 seconds. Probe trial was done on day 6 to assess retention of spatial navigation memory. During the probe trial, the platform was removed, and the rat was again immersed in the water in the opposite quadrant to the platform's location. The rat was allowed to freely swim for 2 minutes and the percent time spent in each quadrant was measured. On the same day, and in order to assess the rats' motor and visual abilities, a visible platform was placed in the pool and the rat was left to swim to reach it. Each rat had four trials with different immersion points. The time needed to reach the visible platform was then measured. All test trials were video recorded and

analyzed using the automated SMART software (Panlab, Harvard apparatus, USA).

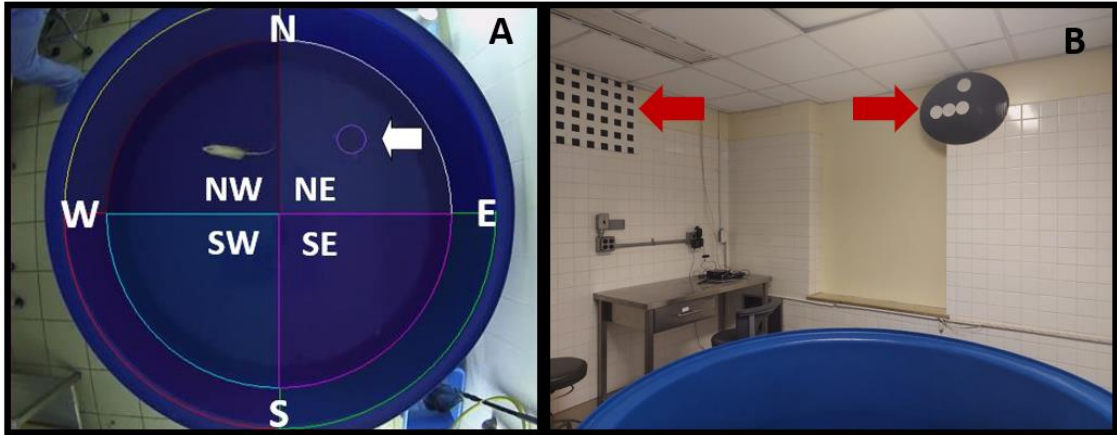


Figure 7 **Morris Water Maze test. Panel A.** The pool is virtually divided into 4 quadrants (NE, NW, SW, SE) where the invisible platform was placed in the north east quadrant. The rat was left to swim freely for 2 minutes per trial to find the escape platform. **Panel B.** The rat uses visual cues hanged on the wall (indicated using the red arrow) for visuospatial navigation. The visual cues were kept constant throughout the test.

##### 5. *Modified active avoidance test (MAAV)*

To assess the ability to learn contextual and auditory cued adaptive shock avoiding behaviors (which is related to the amygdalo-hippocampal circuitry), the MAAV test was performed. This test is developed in our laboratory (63), and consists of a shuttling box (Coulbourn Instruments, Harvard apparatus, USA) that is divided into two equal compartments (H: 34 cm, W: 27 cm, L: 27 cm), and linked by a  $9 \times 9$  cm door located in the middle of a metallic partition wall. The box is placed in a soundproof isolation cubicle (H 80 cm, W 53 cm, L 53 cm) (Coulbourn Instruments, USA) and is equipped with a tone generator (auditory cue, 4 kHz, 86 dB) and infrared beam sensors that detect

transitions between the chambers. The test also consists of habituation (day 0), shock avoidance training (days 1-5), and retention testing (day 6). On the habituation day, rats were allowed to freely move for 5 minutes without any shocks in the shuttling box covered with white foam panels. During the training days, the anterior and posterior walls of the right compartment were changed to striped black and white foam panels with contextual cues (dice and beads) were added on the middle partition wall, while the left chamber was kept as it was on the habituation day. The graphic state 4 software was programmed to give electrical foot shocks (0.5 mA, 15-second duration) with 24 second inter-trial rest period after a signal of 15 second tone whenever the rat enters the left chamber. While in the right chamber, the software was programmed to give an electrical shock every 8 seconds spent in that chamber (Figure 8). Shuttling between the chambers either prevents an incoming shock (shock avoidance) or terminates an ongoing one (escape). A total of 30 trials were done for both chambers. The retention day was divided into two parts. The first part consisted of two sessions, 2 minutes each, where the rat was allowed to freely move in the compartments in each session with no shocks in order to assess retention of contextual learning (chambers were the same as in training days). In the second part of the retention day, the two chambers were covered with white foam panels and visual cues were removed. The rat was given a 5 min habituation, after which 30 tone signaled shocks were delivered following a 15 second tone with a 30 second inter-trial period. Then, a two-minute continuous tone was given to assess freezing responses. The compartments were cleaned between rats on each day with odorless detergent followed by 70% ethanol solution.

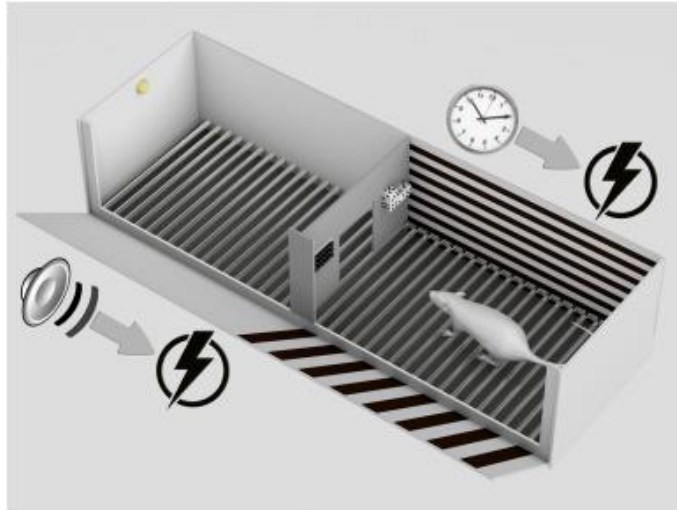


Figure 8 **Modified Active Avoidance test.** A schematic design showing the protocol of the Modified active avoidance test. In the right chamber, the walls were striped and contextual cues were placed. An electrical shock was given for every 8 seconds spent in this chamber. In the left chamber, no contextual cues were added. An electrical shock was given after a 15 second tone in this chamber.

### **E. Sacrifice and Cardiac perfusion surgery**

To perform various histological studies, rats underwent transcatheter perfusion and non-survival surgery 45 minutes after the MAAV test to ensure the detection of learning-induced and freshly expressed plasticity marker protein (Syn), while other rats underwent this surgery a week later for neuronal count (NeuN). After ensuring that proper anesthesia was achieved by the rat's unresponsiveness to noxious stimuli, the animal was pinned from the limbs to a tray under a chemical fume hood with the chest upward, and the abdominal cavity was opened. Then, the heart was exposed by cutting the diaphragm. A needle was then placed on the apex of the heart and the right atrium was cut for fluid drainage. 100 ml of 0.1 M phosphate-buffered saline (PBS) (1X) solution was pumped into the heart to flush the blood out of the circulatory system, followed by 100 ml of 4% depolymerized paraformaldehyde (PFA) to fixate the animal's organs. Then, decapitation was done, and the brains were collected and stored

in 4% PFA for 48 hours, and then in a 30% sucrose solution. They were kept at 4 °C for later paraffin embedding, sectioning, and histological analysis.

## **F. Histological studies**

Coronal sections of 8 µm thickness were obtained. Sections obtained were closest to the KA injection site.

### ***1. Immunohistochemistry***

Immunohistochemistry was done on 3-4 brains per group, and on 6 sections per brain. NeuN and Syp immunohistochemistry was performed to check neuronal count and synaptic plasticity. Slides were de-paraffinized in 3 changes of xylene for 10 min each, then rehydrated in changes of descending concentration of ethanol solutions (100%, 95%, 75%, then 50% of ethanol, 5 minutes each), and washed in distilled water for 5 minutes. After that, slides were incubated in citrate buffer (pH=6) at 90 °C for 60 min for antigen retrieval and then rinsed in distilled water for 5 minutes. After that, the sections were treated for 5 minutes with peroxidase block to neutralize endogenous peroxidase activity followed by a 10-minute washing in PBS. Then, protein block was added to the sections for 5 minutes to reduce non-specific binding of the antibody, followed by another 10-minute washing in PBS. Lastly, the slides were incubated overnight at 4 °C with primary antibody of the following: Anti-NeuN (MAB377; dilution 1:100, Millipore), and anti-Syp (sc-12737; dilution 1:500, Santa Cruz Biotechnology). On the following day, sections were incubated with a post

primary (rabbit anti- mouse) for 60 minutes and a Novolink™ polymer solution (anti rabbit) for 30 min. DAB solution (DAB chromogen with DAB substrate Buffer) was added to the sections and counterstaining was done via Hematoxylin for 5 min. The slides were then rehydrated in two washes of distilled water, for 5 minutes each, followed by dehydration in ethanol solutions, for (75%, 95% and 100%) 5 minutes each as well. Novolink Polymer Detection Kit- 500 Tests (Leica Biosystems, Germany) was used to accomplish these steps.

## ***2. Image analysis***

Immunostained brain sections were imaged using the uSCOPE (uScope MXII, USA) machine. CA1-CA3 NeuN positive cells were counted manually, and hippocampal optical density was measured on Syp stained sections using Image J software (NIH, US). The surface area of the hippocampus was also measured after manually outlining its borders along the ventricles ventromedially and the corpus callosum dorsolaterally with the same software.

## ***3. Statistical analyses***

All data analyses were performed using Prism 8 (GraphPad Software, USA). Animals were randomized to treatment groups prior to seizure induction or any data analysis. The MAAV learning acquisition, FST, and the MWM spatial acquisition data were analyzed using two-way analysis of variance (ANOVA) with repeated measures, followed by post hoc Fisher

least significant difference (LSD) test. MAAV retention, LDT, and OFT data were analyzed using one-way ANOVA with post hoc Fisher LSD test. Histological densitometric measurements and cell count analyses were performed using NIH ImageJ software. A p-value of less than 0.05 was considered statistically significant.

## CHAPTER IV

### RESULTS

#### **A. KA injection and EEG changes post NCSE induction**

Subconvulsive KA doses administered to groups LSK (1 seizure induction) and LRK (2 seizure inductions across 2 consecutive days) induced NCSE in all rats which was confirmed by changes in EEG and behavior. The behavioral changes that were checked for included oromotor automatisms (repetitive motor movements), motionless starrng (confirmed unresponsiveness to tapping on the cage). On the first seizure induction day, latency to seizure between the two groups was comparable (LSK:  $39.86667 \pm 8.341443$  min and LRK:  $44.76471 \pm 10.55397$  min,  $p > 0.05$ , one-way ANOVA), similarly latencies were comparable to the LRK group on day 2 ( $31.375 \pm 4.964772$  min,  $p > 0.05$ , one-way ANOVA). Duration of seizures was comparable between the two groups on day 1 (LSK:  $104.800 \pm 14.60991$  min and LRK:  $154.3529 \pm 26.01752$  min,  $p > 0.05$ , one-way ANOVA), and comparable to the LRK group on day 2 ( $181.3889 \pm 60.19544$  min,  $p > 0.05$ , one-way ANOVA). For the long-term EEG recordings, no seizure recurrence was detected.

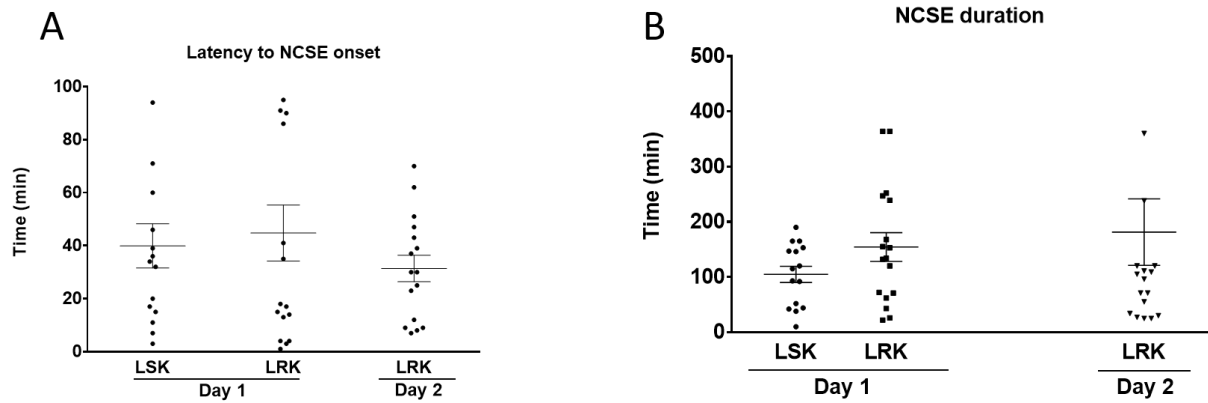


Figure 9 **Induced seizures. Panel A.** The time needed (latency) to NCSE onset post-KA injections was comparable between the LSK group and the LRK group on day 1, and similarly both were comparable to the LRK group on day 2 ( $p > 0.05$ ). **Panel B.** The duration of seizures was comparable between the LSK group and the LRK group on day 1. Duration of both groups was also comparable to the LRK group on day 2 ( $p > 0.05$ ). (LSK  $n=15$ , LRK  $n=16$ ).

## B. Behavioral Results

In order to assess the anxiety-like, hyperactivity and exploratory behaviors, LDT (closed chamber) and OFT (open chamber) were used based on the rodent's natural tendency to explore new objects and illuminated environments. In the LDT, all groups (LCTR, LSK, LRK) spent comparable time in the lit chamber (Figure 10-A). Additionally, the number of entries to the lit chamber was comparable between the different groups (Figure 10-B).

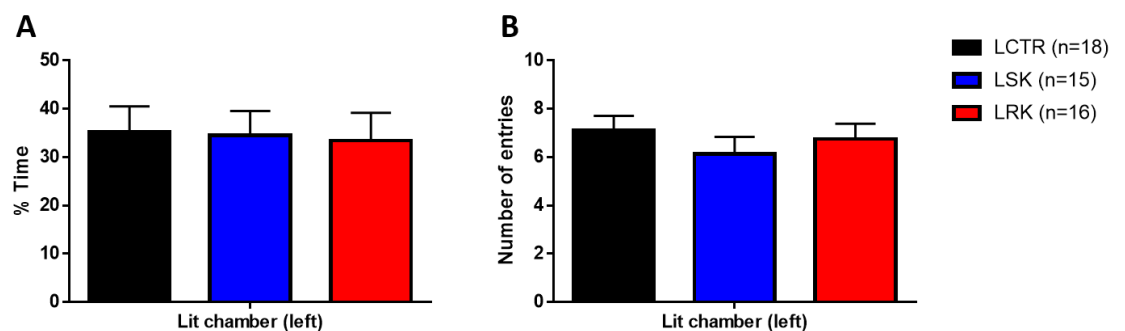


Figure 10 **Results of the Light Dark Test. Panel A.** The time spent in the lit chamber was comparable between the groups. **Panel B.** The number of entries into the lit chamber was comparable between all groups. Mean $\pm$  SEM are reported. (LCTR long term control n=18, LSK long term single kainic acid n=15, LRK long term repeated kainic acid n=16).

In the OFT, all groups travelled a comparable distance over all the 3 sessions (Figure 11-A). All the groups spent an equal duration of time in the periphery in each session shown by comparable percentage of time (Figure 11-B).

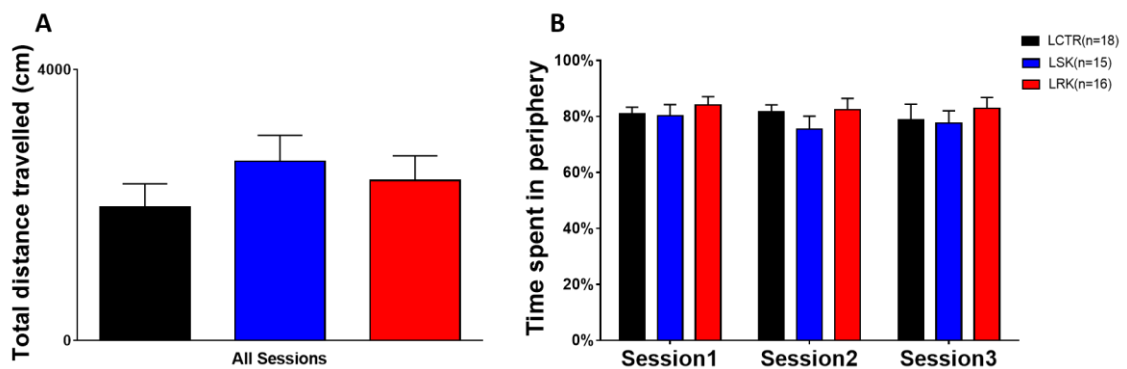


Figure 11 **Results of the Open Field Test. Panel A.** Total distance travelled over all 3 sessions was comparable between all groups ( $p > 0.05$ ). **Panel B.** Time spent in the periphery in each session was comparable between all groups ( $p > 0.05$ ). Mean $\pm$  SEM are reported. (LCTR long term control n=18, LSK long term single kainic acid n=15, LRK long term repeated kainic acid n=16).

The effect of NCSE induction on depressive-like behaviors was also checked by the FST. On days 1 and 2, all groups showed comparable immobility percentage (Figure 12). On both days and for all the groups, the percentage immobility gradually increased from minute 1 to minute 10.

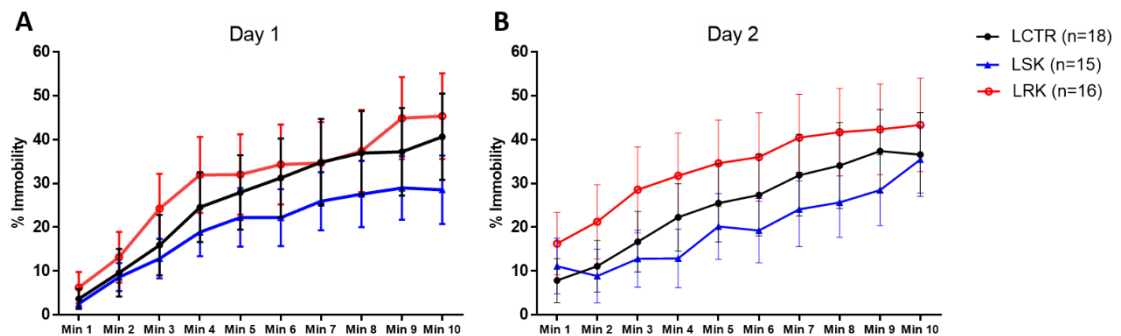
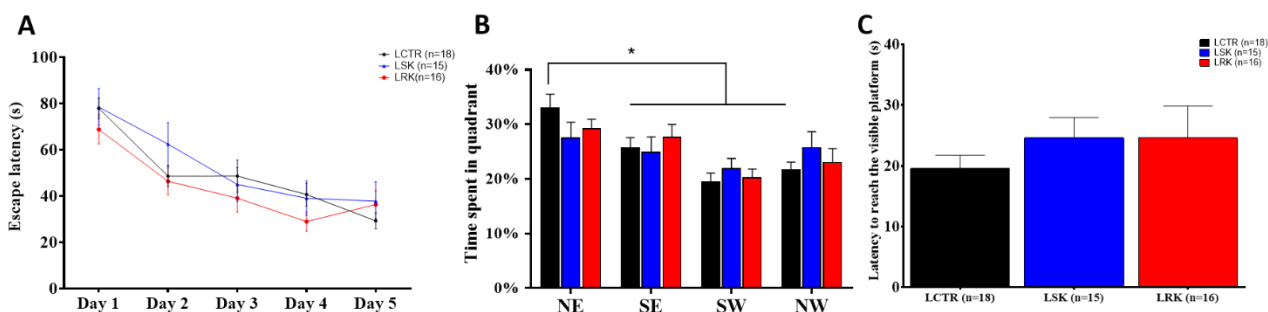


Figure 12 **Results of the Forced Swim Test. Panel A.** Percentage of immobility per minute during swimming on day 1 was comparable between all groups ( $p > 0.05$ ). **Panel B.** Percentage of immobility per minute during swimming on day 2 was comparable between all groups ( $p > 0.05$ ). Mean  $\pm$  SEM are reported. (LCTR long term control  $n=18$ , LSK long term single kainic acid  $n=15$ , LRK long term repeated kainic acid  $n=16$ ).

In order to assess the effects of NCSE induction on visuospatial navigation learning and memory, MWM test was performed. All the rats in all groups learned to reach the escape platform gradually after the 5 training days and results were comparable (Figure 13-A). In the probe trial, the control group spent the highest amount of time in the NE quadrant which was statistically significant compared to the other quadrants ( $p < 0.05$ , one-way ANOVA) while the injured groups spent comparable amount of time between the 4 quadrants (Figure 13-B).

To check for the visual acuity post NCSE induction, a visible platform was placed. Escape latencies to the visible platform were comparable between the groups (Figure 13-C).



**Figure 13 Results of the Morris Water Maze Test. Panel A.** Escape latencies on each day was comparable between all groups ( $p > 0.05$ ). All rats learned to reach the escape platform by day 5 at a similar pace. **Panel B.** The LCTR group had the highest preference to the NE where the escape platform was previously placed. This preference was statistically significant compared to the other groups ( $p < 0.05$ ). **Panel C.** All groups had comparable latencies to reach the visible platform which indicates no damage to visual acuity or motor skills ( $p > 0.05$ ). Mean  $\pm$  SEM are reported. (LCTR long term control  $n=18$ , LSK long term single kainic acid  $n=15$ , LRK long term repeated kainic acid  $n=16$ ).

In the MAAV, percentage of avoidance of tone signals was comparable between all groups across all the days (Figure 14-A). On the day of retention of auditory learning, all groups showed a comparable percentage retention of the avoidance signals (Figure 14-C). In addition, all groups showed comparable percentage of avoidance of context-cued shocks that increased from day 1 to day 5 (Figure 14-B). On the day of retention of contextual learning, all rats spent a comparable time in the left compartment (Figure 14-D).

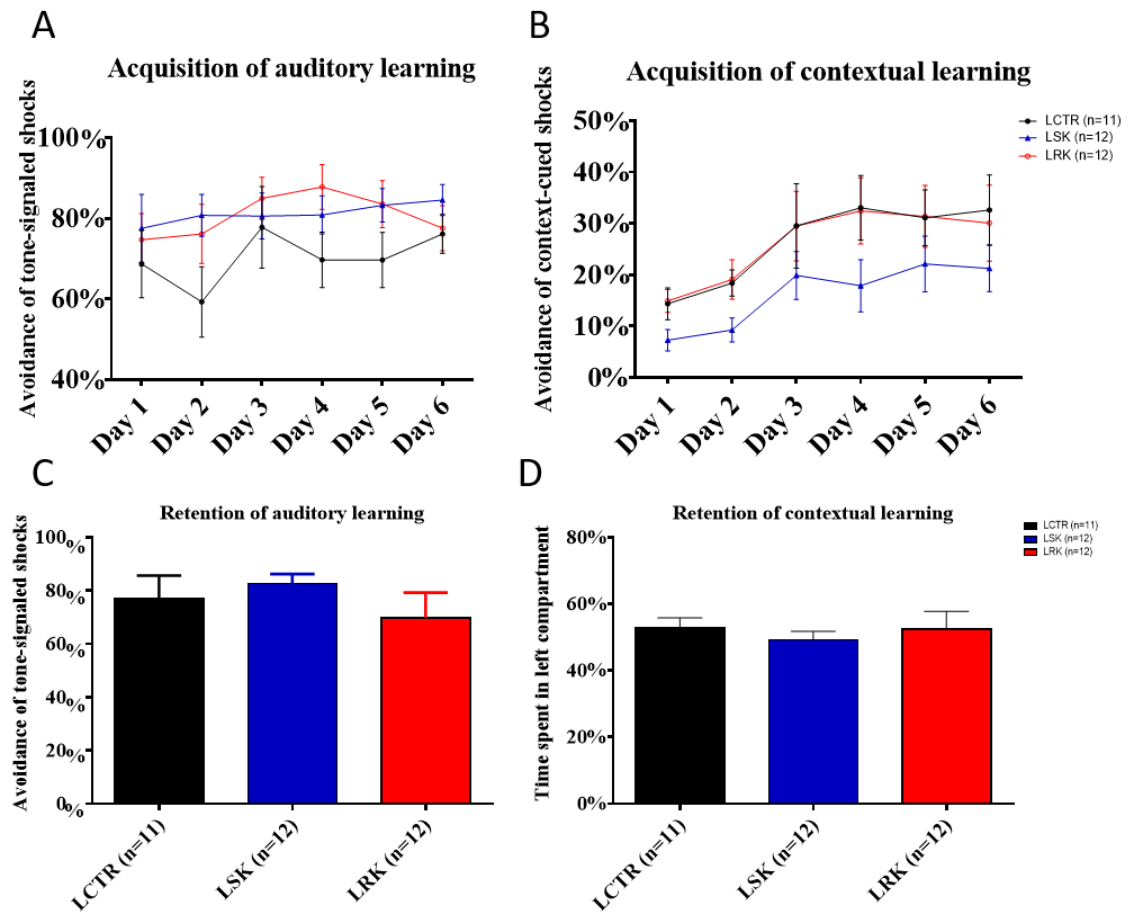


Figure 14 **Results of the Modified Active Avoidance Test. Panel A.** All groups showed same percentage of avoidance of tone signaled shocks which was comparable ( $p > 0.05$ ). **Panel B.** Avoidance of context-cued shocks were comparable among all groups across the 6 training days ( $p > 0.05$ ). **Panel C.** All groups were able to retain auditory learning information shown by comparable percentage of avoidance of tone-signalized shocks ( $p > 0.05$ ). **Panel D.** All groups spent comparable time in the left compartment which shows similar retention of contextual learning among all groups ( $p > 0.05$ ). Mean $\pm$  SEM are reported. (LCTR long term control n=11, LSK long term single kainic acid n=12, LRK long term repeated kainic acid n=12).

### C. Histological analysis

As a marker for mature neuronal cells, neurons were counted and quantified in proportion to the surface area. The preliminary data showed comparable neuronal densities in both the left and the right hippocampi between all groups (Figure 15). ( $p > 0.05$ ; One Way ANOVA with post hoc Fisher LSD)

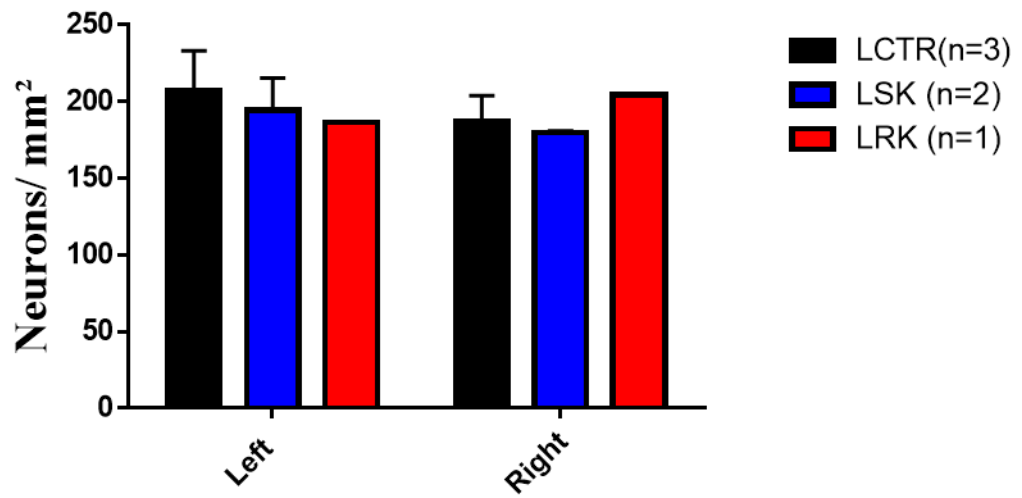


Figure 15 **Hippocampal Neuronal Densities**. Neuronal densities in the left hippocampus were comparable between all the groups ( $p > 0.05$ ). Similarly, the neuronal densities in the right hippocampus showed comparable values between the groups. (LCTR n=3, LSK n=2, LRK n=1)

## CHAPTER V

### DISCUSSION

Unlike convulsive SE, the potential harmful consequences that may result from NCSE remain understudied. Although very few studies report the results of NCSE on the brain, more studies are needed as the knowledge remains insufficient. Here, we showed evidence for memory deficits resulting from NCSE that started in adolescence and persisted in later life.

Our pilot study revealed learning deficits one-month post-NCSE. Indeed, the probe trial test done during the MWM test showed that control rats spent more time searching for the escape platform in the quadrant that previously containing the platform (NE), while the injured groups did not. One episode of NCSE or even two episodes can impair learning and retention. In fact, rats with one or two episodes of NCSE could not retain the position of the escape platform; thus, did not spend time in the corresponding quadrant. This shows that NCSE causes impairments in learning visuospatial navigation later on in adulthood indicating long-term neurodevelopmental functional impairment. This is different from what was previously reported (61), however, this may be due to the use of different tests that have different sensitivities and learning task difficulties.

As for the emotional disturbances, this pilot study showed no long term neurodevelopmental emotional behavior deficits as shown by the results of the LD, OF, and FS tests done one-month post NCSE. Unlike previous studies that cause emotional disturbances and decrease social interaction (61) , our pilot study didn't find evidence

for any of the latter. This may be due to the fact that the social interaction testing is different than the tests done in this study. We also aim at doing sub analysis where we will only analyze the results of the rats that demonstrated the highest duration of seizures. This will help us correlate the duration of non-convulsive seizures in emotional behavioral changes.

Due to the MAAV test's protocol change, the numbers of rats for this test were dropped, resulting in a low number power for this test. The preliminary data did not reveal any long-term emotionally relevant learning and memory deficits, unlike our previous study that showed short-term deficits as the outcome of MAAV (62). However, this data remains preliminary and additional studies are needed to confirm this finding.

Preliminary data of NeuN immunostaining did not show any hippocampal cell loss in the late phase post NCSE. Despite the deficits we show in learning, the neuronal cell count did not show any decrease in the neuronal densities post one or two episodes of NCSE. However, more studies are needed to truly understand the molecular mechanisms of NCSE. However, neuronal cell damage can be subtle and not detected by NeuN cell count. Underlying mechanisms of NCSE-induced learning deficits might involve synaptic dysfunction. This is also in line with some clinical reports show absence of neuronal cell loss post SE which is contrary to popular belief (65), and pre-clinical reports that reveal absence of neuronal cell loss in animal models (60).

## CHAPTER VI

### LIMITATIONS AND FUTURE PERSPECTIVES

This study shows evidence of harmful consequences in the later life that can be attributed to NCSE. Some limitations were faced such as the time restraints. In order for the study to reach enough power for statistical significance, the number of animals should be 15-20 per group. Thus, the behavioral outcomes of the MAAV test are still underpowered due to this low subject numbers. In addition, a minimum of 3 brains (n=3) are needed in the histological NeuN analysis, accordingly our histological data is still preliminary. Another limitation is the lack of intrahippocampal electrode recording. However, our KA injections were given directly to the hippocampus, and the seizure symptoms resemble those of hippocampal origin, which means these seizures are considered as hippocampal NCSE. Finally, for the sake of consistency and due to tight budget, only male rats were used. Maturation and hormonal factors differ between male and female peri-adolescent rats, which affects neuro-development. Future studies will consider using female rodents to investigate the effect of hormonal differences on NCSE, if any. Our lab aims to increase the numbers of animals in the MAAV test to further investigate late life emotional learning post NCSE. Also, further histological investigations are needed to confirm molecular changes post NCSE in line with behavioral changes. In addition, to further investigate potential seizure burden effects, our future studies aim to study the effect of a higher number of NCSE induced episodes.

## CHAPTER VII

### CONCLUSION

As NCSE remains understudied, more patients will be risked to suffer its harsh consequences. Our study shows persistent long-term harmful effects of NCSE on visuospatial navigation and memory. More molecular and histological studies are needed to understand the underlying mechanism behind these cognitive changes. The harmful effects of NCSE shown by this study proves that further research should be done in order to start diagnosing this condition more effectively and calling for better treatment approaches.

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