Effect of Perampanel or Amantadine Treatment on Pilocarpine Rat Model of Status Epilepticus: Evidence with Seizure Termination, Behavioral Alterations, Epileptogenesis and Neuronal Damage.

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By

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ABSTRACT

Persistent activation of ionotropic glutamatergic receptors contributes to seizure sustenance and neuronal cell death. Status epilepticus (SE) was induced in adult male Sprague Dawley [12 to 14 weeks old] rats by treating them with pilocarpine. The efficacy of either perampanel, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker, or amantadine, a N-Methyl-D-aspartic acid (NMDA) receptor blocker, in overcoming pilocrapine-induced SE was assessed using electroencephalogram (EEG) recordings. In addition, the alterations in cognitive function, development of spontaneous recurrent seizures (SRSs), and hippocampal damage that are generally encountered in SE were also assessed at 72 hours and 1 month after pilocarpine treatment. Our results show that both early and late treatment with perampanel but not amantadine significantly reduced the latency in the termination of seizure as confirmed by EEG recording. Perampanel but not amantadine, reversed the memory impairment in SE rats and retarded the appearance of SRS. Fluoro-Jade C staining and NeuN immunohistochemistry revealed the protective effects of perampanel. Perampanel treatment led to reduced caspase-3 activation in the hippocampal sections of brains isolated from SE rats. In vitro addition of either perampanel or amantadine in primary cultures of hippocampal neurons significantly reduced the levels of cytotoxicity and caspase-3 activation induced by AMPA and NMDA. Both perampanel and amantadine treatment also reduced GAPDH, p53, PTEN, and active SREBP-1 levels expressed in nuclear fractions isolated from the primary cultures of hippocampal neurons treated with either AMPA or NMDA. Our data might shed some light in the therapeutic approach of perampanel in clinical use for status epilepticus.

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BSA	Bovine serum albumin
CF	Cytodolic fractions
DAG	Diacylglycerol
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCRs	G protein-coupled receptors
IBE	International Bureau for Epilepsy
IP3	Inositial triphosphate
MTLE	Mesial Temporal Lope of Epilepsy
NF	Nuclear fractions
NMDA	N-Methyl-D-aspartic acid
PIP2	Bisphosphate
PLCβ	Phospholipase C-beta
PTEN	Phosphatase and tensin homolog
SCD1	Stearoyl-CoA desaturase-1
SE	Status epilepticus
SREBP-1	sterol regulatory element-binding protein 1
SRSs	Spontsnous recurrent seizures
TBST	Tris buffered saline with triton

Dedication

I dedicate this work for my beloved family

1. Introduction

1.1. Status Epilepticus

Status epilepticus (SE), is a life-threatening emergency that is often referred to as the "extreme expression of epilepsy". The original definitions of SE have evolved into a more narrow and specific description that reflects the seriousness of this disease. In 1981, the European Electroencephalographic Meeting Defined status epilepticus as seizures that continues for a sufficient length of time or are repeated frequently without recovery of consciousness between attacks. These concepts, while highly valuable, were inaccurate, as they did not define the duration of a seizure. Over the past two decades, the timeline in status epilepticus related clinical trials and treatment recommendation moved to 30 min in 1993 (1) then to 20 min in 1998 (2) and to 10 min in 1999 (3). Lowenstein in 1999 recommended that a generalized tonic-clonic seizure that persists 5 minutes is a prolonged seizure and has to be treated as SE (4). The International League Against Epilepsy and the Commission on Epidemiology came out in 2015 with the following definition: **"Status epilepticus is a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms, which lead to abnormally prolonged seizures (after time point t1). It is a condition, which can have long-term consequences (after time point t2), including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizures" (5)**. In general, time point t1 is the time when treatment for SE should be started, which is at 5 min for generalized tonic-clonic status epilepticus, and at 10 min for focal seizures with or without impairment of consciousness. Time-point t2 marks the time at which neuronal damage or sustained

alteration of neuronal networks may begin, and indicates that SE should be controlled by that time the latest; 30 min in case of generalized tonic clonic seizures.

1.1.1. SE epidemiology and its high morbidity and mortality despite treatment

Most epidemiological studies have used a traditional 30min duration of seizure to define SE, and so the estimates of SE given in those studies are the lowest (currently is 5 min internationally). Using the 5min definition, determining the time from the beginning of the seizure to starting emergency treatment, the rate in clinical practice has to be much higher than in the rate reported in the epidemiologic studies. The overall incidence of SE is 9.9 to 41 per 100,000/year worldwide, with peaks among children and elderly (6). It is estimated that there are 54,000 seizure-related visits to emergency department per year in Canada, and as many as 3,800 cases are due to SE (7). Of these, almost 25% (e.g. 850) result in death (8). Mortality is not evenly distributed across age groups; indeed, the mortality is higher in the elderly (60 years and above) at 38% compared to younger adults (16-59 years) at 14% (8). Furthermore, SE is three times more common in the elderly. Given that the population of Canada and Saskatchewan is aging, SE is likely to become a significant social, economic and clinical burden. The morbidity of SE, including permanent learning and memory deficits, is seen in up to 70% of patients (9). It is known that prolonged and refractory SE is associated with poor outcome (10). The persistent high morbidity and mortality in SE demonstrate that current treatments are not fully effective and that improved treatments for SE are critically needed. Any new treatment(s) will have to protect neurons from damage induced by the ongoing seizures as this will surely improve clinical outcomes, including the preservation of normal learning and memory abilities.

1.1.2. Etiology

In adults with chronic epilepsy, the most common causes of SE are low levels of the antiepileptic drug, remote symptomatic causes, and stroke (11). In general, approximately 48-63% of all SE cases are due to acute symptomatic causes such as SE that occurs after metabolic insult, certain infections in the Central Nervous System (CNS), stroke, brain trauma or cerebral haemorrhage (12). In the Richmond Virginia Status Epilepticus Study, 41% of adults and 61% of the elderly had acute or remote ischemic and haemorrhagic strokes as a cause of SE (9). The primary determinants of the mortality and morbidity associated with SE: (1) certain etiologies, (2) age >60 years, and (3) prolonged SE (13) (14).

1.1.3. Mesial Temporal Lobe of Epilepsy (MTLE)

The Temporal Lope of Epilepsy (TLE), is the most common type of epilepsy in adults, can be associated with memory deficits and cognitive problems (15). Most patients with TLE have experienced a neurological insult that leads to the development of epilepsy. Status epilepticus is a major example of brain injury that can lead to the development of TLE (16).

The main features of TLE are: (1) the localization of seizure origin in the limbic region, particularly in the hippocampus, entorhinal cortex and amygdala (17); (2) the frequent finding of an initial precipitating insult (IPA) that precedes the appearance of TLE; (3) a latent period during which epileptogenic process takes place, which in turn lead to spontaneous recurrent seizures (SRSs); (4) a high incidence of hippocampal sclerosis, i.e.; segmental loss of pyramidal neurons, dispersion of granular cells and reactive gliosis (18) (19) (20) (21), (5) a high prevalence of behavioural disturbances and cognitive impairment (22) (23) (21) (24) (25) (26), (6) MTLE patients are among the most refractory to medical treatment (27).

Most of the characteristic features of TLE can be replicated in chronic animal models of TLE, especially kindling and status epilepticus animal models such as the pilocarpine and kainite animal models of SE (28). For the purpose of the current study, we used the pilocarpine rat model to recapitulate the events of status epilepticus and the chronic development of temporal lope epilepsy. This model appears to be highly isomorphic with the human TLE. The pilocarpine model has been used in many laboratories since its description in 1983 to investigate the pathogenesis of TLE and to evaluate the efficacy of anti-epileptogenic drugs (reviewed in: (29, 30).

1.2. The pilocarpine rat model

The pilocarpine model was first described in 1983 in rats (31) (32) and then in mice (33) to produce limbic seizures. This model is widely used and has been modified in many laboratories. Depending on the aims of the experiments, different laboratories use different dosages of pilocarpine, pre-treatment protocols and animal species (29, 30). In addition, the duration of SE induced by pilocarpine or the anti-epileptic drugs used to terminate the seizure varies as well. Pilocarpine can induce SE in rats after systemic (34), intracerebroventricular (35) and intrahippocampal administration (36). For the systemic route of administration, the pilocarpine dose necessary to induce SE ranges from 300 to 400 mg/kg in adult rats (37, 38). Lower pilocarpine doses (100 - 200 mg/kg) may produce brief and less severe seizures, but do not result in SE (32). The dose of pilocarpine administered to induce SE significantly affects the latency to seizure, mortality rates, and neuropathology. When compared to lower doses of pilocarpine (300 mg/kg, i.p. or less), higher doses (350 – 400 mg/kg, i.p.) have resulted in a reduced latency to SE onset and a greater percentage of rats developing SRSs (37, 38). However, higher doses of pilocarpine

have also resulted in greater mortality rates, and this may be caused by the more intense seizure and brain damage (37, 38).

1.2. 1 Pilocarpine initiates seizure through the activation of M1 Receptors

The systemic administration of the potent muscarinic agonist, pilocarpine, initiates seizure through the activation of the muscarinic M1 receptors. Other cholinergic agonists, such as carbachol and Bethanechol can induce seizure and seizure induced brain damage in rodents as well (39). Furthermore, the pilocarpine induced-SE can be antagonized by the systemic administration of the non-selective muscarinic antagonist, atropine (32, 40). Moreover, M1 knockout mice do not develop status epilepticus after pilocarpine administration (39).

M1 receptors are G protein-coupled receptors (GPCRs), which are in turn coupled to the activation of phospholipase C-beta (PLCβ) (41). PLCβ dependent cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) generates inositol triphosphate (IP3) and diacylglycerol (DAG) (Berridge, 2009), resulting in an alteration in a Ca2+ and K+ current and increasing the excitability of the brain (42). An alternative mechanism by which M1 receptors may generate excitatory action involves Src kinase activation (43). Activation of M1 receptors can increase the intracellular Ca2+ levels, which stimulates Src kinase activation (44). Src kinase phosphorylates other signaling molecules, including soluble guanylyl cyclase and extracellular signal-regulated kinase (ERK) (43), both of which have been implicated in cholinergic excitation (43, 45). In addition, in vivo microdialysis studies have revealed that pilocarpine induces an elevation in glutamate levels in the hippocampus following SE (46).

Over the past two decades, research has elucidated a serious of maladaptive changes that contribute to the transition from a single seizure initiated through muscarinic receptor activation

to status epilepticus. As mentioned in the above paragraph, in-vivo microdialysis has shown that pilocarpine induces an elevation in glutamate levels in the hippocampal formation after the induction of seizures (46, 47). The glutamate propagated neuronal excitability can accelerate the internalization of the inhibitory Gamma-Amino Butyric Acid A (GABAA) receptors (48). Once seizures are initiated, their maintenance depends on mechanisms different in nature from the muscarinic receptors, since muscarinic antagonist become ineffective in terminating established seizures (49). The elevated glutamate levels suggest an excessive excitatory drive during the acute phase of pilocarpine model. Substantial evidence now supports the idea that following the activation of muscarinic receptors, SE is maintained by activation of glutamate receptors (50).

1.2. 2 Behavioral and clinical features of seizure development in the pilocarpine model

The epidemiologic study reported that up to 42% of patients who experienced SE as their first seizure (mean age 39.7) developed epilepsy in the form of TLE over the next 10 years (51). The pilocarpine model closely mimics some important features of TLE, in which an initial precipitating brain insults like SE, is frequently followed by a latent phase followed by the development of recurrent seizures. An acute episode of SE serves as the IPI (29, 30). The three stages of seizure development in response to pilocarpine administration are described below. These are the acute phase, epileptogenesis, and the chronic phase.

1.2.2. 1 The acute phase

Following the pilocarpine administration, rodents experience a series of frequent seizures, including SE.

i. Behavioral seizures during the acute phase

According to Turski et al. (1983a), behavioral manifestations increase with time following pilocarpine till the development of generalized tonic clonic seizure. After 5-10 min of pilocarpine intraperitoneal injection, animals were motionless followed by peripheral cholinergic stimulation symptoms such as salivation, piloerection, eye blinking and urination. During this period animals are able to be distracted by tactile or auditory stimulation (52). These cholinergic symptoms remain to be observed for approximately 45 min following pilocarpine injection. Then subside as the limbic motor seizure in the upper extremity clonus start to develop within 30-40 min after pilocarpine injection. Four limb clonus, rearing and falling, typically develops soon following the initial limbic seizures. During SE seizure, rats are unresponsive to external touch and sound stimulation (37). In the absence of specific measures to terminate SE, seizure spontaneously continues within 5 to 6 hours (32, 37). Animals lose their consciousness for the 24 hour period following cessation of SE. Body weight decreased after SE (10–20%) but recovered quickly after approximately 1 week (see figure 13).

Mortality rates after pilocarpine induced status epilepticus have been reported to be around 30–40% for male Wistar rats treated with 300–400 mg/kg pilocarpine (31, 53). As an alternative approach to reducing mortality, we have aimed to terminate the SE duration to 60-90 min using pentobarbital. Indeed, reduction of the SE length does result in a significant decrease in mortality rate. However, it must be noted that SE duration of 60-90 min is crucial for the development of the full syndrome including SRS. Furthermore, pentobarbital is widely used to limit SE duration

in post-SE models of TLE in order to reduce SE-associated mortality and to avoid the variability in long-term consequences of SE arising from differences in SE duration among individual rats (Goodman, 1998). When the SE duration is of sufficient length to produce neuronal damage and development of epilepsy, pentobarbital does not seem to exert any obvious neuroprotective effect.

ii. Electrographic activity in the acute phase of pilocarpine rat model of SE.

EEG recording after pilocarpine produces both ictal and interictal epileptiform correlate well with the behavioral changes described in section 1.4.1.1. Immediately following pilocarpine injection, low-voltage, fast activity reading appears in the cortex while a clear theta rhythm appears in the hippocampus. As the behavioral features of SE become more intense, high voltage, fast EEG activity take over the hippocampal theta rhythm. Electrographic seizures characterized by high voltage, fast activity and prominent spiking precede limbic motor seizures, and are proposed to result from M1 receptor activation (54). It has been proven that this activity arises in the hippocampus and spread to the cortex(32) (53) (55). If not terminated, sustained electrographic activity lasts 4-5 hours followed by periodic discharges on a relatively flat background.

iii. Scoring of pilocarpine induced seizures

In this thesis, we used Racine criteria (56), which at first was developed to score kindled seizures in adult rats. The seizure scoring was as follows: Stage 1, immobilization, eye blinking, twitching of vibrissae and mouth movements; Stage 2, head nodding, often accompanied by severe facial clonus, piloerection; Stage 3, straub tail, forelimb clonus; Stage 4, rearing; Stage 5, rearing, falling and generalized convulsions some animals may develop wild running and jumping with vocalization.

1.2.2. 2 The latent phase

The latent phase also referred to as epileptogenesis, is the period between the brain insult and the onset of spontaneous seizures. During this stage, animals show normal behavior and no seizure activity. It is believed that during the latent period, pathophysiological changes take place within the neurons and end up by the appearance of spontaneous seizure (57). The duration of the latent period varies depending on the experimental protocol used. Several research have examined the latency to develop SRS. After a high single systemic dose of pilocarpine, the latent period range between 4-44 days in different species of rats (58, 59).

A minimum duration of SE is required for the development of chronic epilepsy. For instance, using the pilocarpine rat model, Lemos and Cavalheiro (1995) were able to detect SRS in rats experienced SE duration of 60 min, but not in rats with only 30 min of SE (60). Furthermore, Biagini (2006) found that the rats experience a short SE duration 1-2h had a progressively shorter latent period than animals with longer SE (3-6h), and this was suggested to be related to the increased levels of neurosteroids produced by glial cells. Since neurosteroids, such as allopregnanolone, act as modulators of the inhibitory Gamma-Amino Butyric Acid A (GABAA) receptor, which may delay the appearance of SRSs (61).

1.2.2. 3 The chronic phase

The chronic phase pilocarpine rat model starts by the appearance of SRSs, which continues for the lifetime of the animal, same happens in patients who develop TLE after a severe brain insult such as SE.

i. Behavior during the chronic phase

The behavioral observations of pilocarpine-induced seizures can be classified by referring to stages 1-3 as partial seizures, and stages 4-5 as tonic component (62). The seizure seen during SRS are usually characterized by facial automatism, four limb clonus, rearing, falling followed by tonic clonic seziures. Once SRSs (stage 5 seizure) appears, they start to recur in clusters with cyclicity, peaking every 5 to 8 days (62). The frequency of SRSs increases within the first weeks of SRS appearance and then remains constant 2 months after SE and persists throughout the lifetime of the animal (62, 63). Aggressive and anxious behaviour is usually observed before the beginning of SRSs and wane during the seizure period. In addition, rats with aggressive behavior tend to develop early-onset spontaneous seizures and are also likely to have refractory seizures (64). Immediately after the seizure, animals are usually frightened and may experience drawsiness.

ii. Electroencephalographic patterns observed during SRSs

The first spontaneous seizures are partial seizures characterized by paroxysmal activity in the hippocampus that does not appear in cortical recordings (65). Subsequent seizures show a gradual spreading of paroxysmal activity from the hippocampus to cortical recordings and longer duration of ictal events. The fully developed generalized seizures are characterized by bursts of spiking activity in the hippocampus that spread to the cortex (65, 66). Electrographic seizures during RSR unusually last more than 60 sec and are followed by depressed background activity with frequent interictal spikes.

1.3. Histopathological changes of SE induced TLE

1.3.1. SE-induced neurodegeneration

Status epilepticus is consistently associated with widespread brain damage in the hippocampus and other brain regions. Most of the neuronal damage happens in the few days following SE (67, 68). It is not clear weather SRSs contribute to the neurodegeneration in the chronic period, or the detected neuronal loss in this stage is due to a chronic effect from the initial SE. While no connection between the neuronal injury and the frequency of SRSs was reported in some studies(69) (57, 70), other studies showed a more progressed loss of neurons in the chronic stages after SE (71).

The majority of patients with TLE develop epilepsy after brain damage secondary to an initial precipitating insult such as, SE (19). While some TLE patients show no obvious brain lesions, approximately 70% shows hippocampal sclerosis. All of these features were found in specimens resected from patients undergoing surgery for medically intractable TLE, and it is characterized by; (1) loss of pyramidal cells and synaptic reorganization, (2) mossy fiber sprouting and granular cell dispersion, (3) gliosis, (4) dysfunction of the blood-brain barrier (5) and neuroinflammation (72, 73) (74) (75). A similar pattern of neuronal damage is seen the pilocarpine rat model (76) (77). The most obvious change in hippocampal sclerosis is the significant pyramidal cell loss in the hippocampal subfield CA1 and CA3, with lesser extent of neuronal loss in CA2 (78) (79). In the dentate hilus, many excitatory mossy cells are lost (18) (80). Granular cell layer of the dentate gyrus show lesions in 50 % of TLE patients.The lesions in this structure range from mild changes such as granular cell dispersion to severe damage such as a significant cell loss (81) (82). In addition, reorganization of the inhibitory neuropeptide Y (NPY) fiber networks in the dentate gyrus have been reported (83).

1.3.2. Reactive gliosis

Neuronal cell death is accompanied by extensive gliosis in the epileptic brains of TLE patients (84). This phenomenon is the same in animal models of TLE. Reactive gliosis is characterized by hypertrophy of astrocytes exhibiting upregulation of the intermediate filament protein, as well as a proliferation of astrocyte and microglia (85). Astrocytes perform a variety of tasks including, synaptic formation and homeostasis, regulation of neuronal glutamate subunit expression, regulation of blood brain barrier and blood flow, neuronal ionic homeostasis, neurogenesis and neuroprotective properties (86). Alterations in astrocytic properties have been best described in the specific case of human temporal lobe epilepsy. Numerous studies reported the role of glial cells in epilepsy are available (87) (74) (88). Some studies showed that reduced or dysfunctional glial glutamate transporters GLT-1 in the hippocampus increase extracellular glutamate levels and may contribute in triggering SRSs in patients with temporal sclerosis (89). In addition, astrocytes were found to participate in the delayed neuronal damage following pilocarpine induced status epilepticus (90). The seizure induced rise in the astrocyte intracellular Ca2+ operates Ca2+-dependent ion channels and induce glutamate release from astrocytes (91).

1.4. Cognitive deficits after SE

Cognitive impairment problems are common in patients with epilepsy (92). A survey by the International Bureau for Epilepsy (IBE) found that 44% of patients with epilepsy have difficulty in learning and memory formation, 45% felt that they were slow thinkers, 59% felt sleepy or tired, and 63% of patients receiving antiepileptic drug (AEDs) found that the side effects of AEDs prevented them from achieving normal activities (93). The origin of those deficits remains

uncertain. However, several contributing factors have been proposed, including antiepileptic drugs (94) seizures (95), abnormal epileptiform charges (96) and neuronal circuit reorganization (97). Cognitive deficits may include difficulty in learning and memory retrieval (98), in addition, other comorbid psychiatric disorders profoundly impact this patient population such as depression, stress, aggression and anxiety (99).

Impairment of learning and memory has been described in adults and children following SE (100), and in patients with TLE (101). More specifically, patients with TLE frequently showed deficits in declarative memory (ability to acquire facts and events related to one ‘s personal past, which is often compared with visual-spatial learning in rats) (102), and in the performance of visuospatial tasks (103).

Prolonged seizures lead to neuronal cell death and permanent changes in hippocampal anatomy. The physiology of the limbic system, and particularly the hippocampus, plays a crucial role in episodic and working memory. Within the hippocampus, local field oscillations in the theta frequency range (5–12 Hz in the rat) are critical in the formation of short-term and episodic memory (104), as well as spatial working memory (105) and spatial memory (106).

The development of chronic epilepsy induced in rats by systemic administration of pilocarpine reproduces most clinical and neuropathological features of human TLE (32). Previous studies demonstrated severe impairment of hippocampus-dependent spatial memory in pilocarpine-treated rats early after the induction of seizure (107), at the latent period (108) (107), and during the chronic period (109) (110) (111).

1.5. Mechanisms underlying SE- induced cell death

1.5.1. Glutamate and neuronal injury in SE

Status epilepticus induced cell death is believed to be initiated by excitotoxicity (112). Excitotoxicity occurs when glutamate receptors are excessively stimulated. Glutamate receptors are generally divided into subtypes referred to ionotropic and metabotropic. Of the ionotropic receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-Methyl-D-aspartic acid (NMDAR) seem to play a significant role in SE (113). Importantly, the AMPAR and NMDAR are also critical for normal learning and memory formation (114), as they stimulate the synaptic plasticity to maintain memory circuits in the brain (115). Plasticity depends on glutamate receptor trafficking to postsynaptic membrane, where they can participate in synaptic activity (115). SE increases the glutamate levels in the hippocampus after seizure (89). SE increases the turnover and trafficking of glutamate receptors, particularly NMDA and AMPA receptors, to the postsynaptic membrane (116) (117). This leads to rapid calcium influx and a significant increase in intracellular calcium levels. As a result, several calcium-dependent enzymes including neuronal nitric synthase, phospholipases, endonucleases, and cysteine proteases (i.e., calpains and caspases) are activated by SE in an unrestrained manner. These enzymes subsequently damage cell structures such as components of the cytoskeleton, membrane and DNA (118) (119). Furthermore, this excess intracellular calcium activates several signaling pathways leading to mitochondrial swelling, low ATP levels, and accumulation of reactive oxygen species (ROS) (116). In addition, due to dysfunctional mitochondria, a large amount of glycolysis occurs in SE along with overproduction of lactic acid (120). Any accumulation of lactic acid will produce cerebral lactic acidosis, which leads to further increases in the production of ROS and more

mitochondrial dysfunction (120). Eventually, excessive calcium and ROS lead to the collapse of mitochondrial membrane potentials, the activation of mitochondrial matrix enzymes, and the opening of mitochondrial permeability transition pores, which decreases ATP production and induces neuronal cell death. As glutamate is the primary excitatory neurotransmitter in the brain, associated receptors have been deemed a logical target for antiepileptic activity. As glutamate receptor activation occurs at the beginning of this cascade of deleterious events, we propose that targeting glutamate receptors in SE with antagonists would protect from the SE-induced neuronal cell loss.

1.5.2. AMPA receptors (AMPAR) and SE

AMPARs are the principal molecular units for fast excitatory synaptic transmission in the central nervous system and are composed of four subunits, GluR1–GluR4 (121). High expression of GluR2 favors the formation of calcium-impermeable AMPARs. Any combination of GluR1/-3/-4 subunits that lacks GluR2 is calcium-permeable, which increases sensitivity to the excitatory effects of glutamate (Santos et al., 2009). This forms the basis of the "GluR2 hypothesis", which predicts that a relative reduction in the expression of GluR2 enhances calcium influx through newly synthesized AMPAR, thereby increasing neurotoxicity of endogenous glutamate (122). Recent observations have revealed a reduction in GluR2 expression and the formation of calcium-permeable AMPAR's in hippocampal neurons in a model of SE (123). It is also shown that AMPAR-mediated excitation is progressively increased during SE (116). Furthermore, status epilepticus was found to potentiate AMPAR-mediated excitatory transmission of CA1 pyramidal neurons followed by increased surface expression and dephosphorylation of the GluA1 subunits (124).

1.5.3. NMDA receptors (NMDAR) and SE

In many in vivo and in vitro animal models, the use of pilocarpine induced over expression of NMDA subunits in hippocampal neurons (125). It was reported that NR2A containing NMDARs activation was required for the development of limbic epilepsy in kindling and pilocarpine models (126). NMDAR overexpression leads to neuronal hyperexcitability and the prolongation of seizures. Therefore, NMDAR antagonists remain highly efficient in ending SE, even late in its course (127). NMDA receptor antagonists administered to rats before or after SE provide significant neuroprotection (52) (128). In an in-vitro model of SE, specific entry of Ca2+ through NMDA receptors results in more cell death as opposed to Ca2+ entering through non-NMDA glutamate receptors or voltage-gated calcium channels (50). Similar findings have been demonstrated in other models of glutamate neurotoxicity (129).

1.6. Mechanism of cell death initiated by SE

Although it is generally acknowledged that neuronal loss following SE is excitotoxic in nature (50), it is not clear whether the widespread neuronal cell death phenotype after SE is mainly necrotic or apoptotic in nature (112). A detailed discussion mechanism of neuronal death is beyond the scope of this thesis, but various reviews of this topic are available (130) (131). Briefly, necrosis results from severe mitochondrial dysfunction and cell membrane rupture. Therefore, necrosis refers to morphological characteristics seen after the cell has already died and reached into equilibrium with the surroundings (131).

Apoptosis is a distinct morphological type of cell death characterized by leakage of the cellular membrane, degradation of DNA nucleosome, the formation of condensed chromosome and formation of membrane-bound apoptotic bodies (132). Apoptosis and/or necrosis can be initiated by a countless number of neurotoxic signaling pathways, and numerous reviews of these pathways

are available (133) (134). This part briefly explains the roles of caspases and calpains in apoptosis and necrosis. Apoptosis is mediated by specific caspases which can be activated by either intrinsic (receptor-mediated) or extrinsic (mitochondria-mediated) pathways (135). After receiving a death stimulus, the initiator caspases (e.g., caspase-8 and caspase-9) activate some effector caspases such as caspase-3, which in turn stimulate a programmed signaling pathway end up by apoptosis (135). On the other hand, elevated intracellular Ca2+ activated calpain enzymes which stimulate the cleavage of multiple cytoskeletal proteins, kinases, phosphatases, membrane receptors and transporters. The breakdown of cytoskeletal proteins accelerates cell death and contributed to the characteristic morphology of necrotic cells (136). However, assuming the involvement of caspases and calpains to a specific cell death morphology is not straightforward. Alternatively, a widely more accepted view is that excitotoxic cell death is a mixed form of necrosis and apoptosis (137) (119).

The morphological features of cell death after status epilepticus imply that cells die of necrosis (112). Regardless of the primary necrotic morphology of dying neurons after SE, recent studies found that DNA degradation, chromatin condensation and fragmentation, activation of caspases were observed, indicating dying neurons also exhibit apoptotic features (138). In many instances, a heterogenous form of neuronal death with apoptotic and necrotic features following SE were reported (139) (140). Autophagosomes and secondary lysosomes were also detected in dying neurons after SE, indicating that autophagy contributes to neuronal death following prolonged seizures as well (141). Autophagy is usually initiated with increased oxidative stress (142).

1.7. The role of neurodegeneration in epileptogenesis

Prolonged seizures result in widespread injury to the brain. However, the contribution of neuronal loss in the development of epilepsy remains debatable (143). Two points suggest that neurodegeneration after SE is not needed for the development of SRSs. First, numerous studies indicated that complete neuroprotection of the hippocampus, amygdala and cortical formations did prevent the development of epilepsy after a brain insult. For example, the administration of MK801 90 min after kainite induced-SE can prevent most of the hippocampal and para-hippocampal degeneration but did not prevent the development of epilepsy (144). Similarly, a prolonged treatment with valproate was successful in preventing cell loss but has no effect on the development, severity or the frequency of SRSs (16).

Second, many animal models of epilepsy can lead to the development of SRSs with no significant neurodegeneration. For example, prolonged electrical stimulation of specific brain region such as the hippocampus or the amygdala can lead to the development of SRSs after a latent period without neuronal loss. In addition, febrile seizures in immature rats can lead to SRSs without neuronal death (145). Third, it has been observed that SRS in some patients with TLE were successfully controlled by partial removal of hippocampal tissue that exhibited no neuronal loss (146) (147). However, some studies suggest that removal of hippocampal tissue might disrupt the critical neuronal pathways responsible for epileptogenesis induced by neuronal loss in other parts of the hippocampus or other regions of the brain (148).

Despite that, some studies found that SE-induced neuronal loss aggravates the process of epileptogenesis. For instance, Gorter et al., (2001) investigated the development of spontaneous seizure in a rat model after a long electrical stimulation. 67% of the rats that underwent SE, developed SRSs after a latent period of 1 week. The remaining rats did not develop that progression

of seizure. Gorter compared weather different pattern of SRSs development could be related to neuronal changes in the hippocampus. Though both groups underwent SE, the majority of cell damage, decreased parvalbumin and somatostatin immunoreactivity and progressive mossy fiber sprouting were seen among the group that developed SRSs with in short latent period (149). Similar findings that correlate neuronal loss and the development of epilepsy were found in other studies (150) (151). Therefore, it remains possible that neuronal loss is a mechanism among many, that underlie the genesis of TLE; for example, neurogenesis (152), changes in the balance of excitatory and inhibitory processes (153), or alterations in the release or postsynaptic effects of neurotransmitters (154). Thus, although it seems that neurodegeneration is not essential for the development of epilepsy, neuroprotective strategies may have a role in modifying the disease outcome (155) (156) (157).

1.8. Decreased benzodiazepines efficacy with seizure progression in SE

Status epilepticus is a commonly encountered medical emergency (158). SE quickly becomes self-sustaining, independent of its initial trigger and resistance to multiple antiepileptic drugs (9). While researchers have made considerable progress in controlling epilepsy, status epilepticus remains a therapeutic challenge that still carries a 27% mortality and a high morbidity. Despite the treatment guidelines, many SE sufferers end up with permanent brain damage, especially in the limbic system, resulting in memory impairment, cognitive deficits, epilepsy and other neurological morbidities.

The current first-line therapy for SE is based on compounds that potentiate the inhibitory GABAA receptor complex (such as, diazepam, lorazepam. Midazolam and phenobarbital) or

blocks Na+-channels (e.g., phenytoin, fos-phenytoin). However, this first line treatment is effective in discontinuing SE in merely up to 65% of patients (159).

Time-dependent pharmacoresistance is a major therapeutic problem in SE. As seizures continue, pharmacoresistance develops progressively. The anticonvulsant potency of benzodiazepines can decrease 20-fold in 30 min of seizures (160). In adult animals, diazepam was effective in controlling seizures in all animals when it was given 10 minutes after the onset of SE but failed after 45 min. Phenytoin and barbiturates also lose potency, but more slowly (161). Studies have revealed that GABAR-mediated inhibitory synaptic transmission is reduced in the hippocampi of animals in SE, due in part to the internalization of synaptic GABA receptors (162) (163).

As previously described in section 1.5, excitotoxicity mediated via the glutamate receptors is recognized as a major mechanism in neurodegeneration resulting from SE. Consistent with a critical role of NMDARs in SE-induced neurodegeneration, previous studies showed that systematic administration of NMDA antagonists is neuroprotective in rodent models of epilepsy, even when given after the onset of SE (164) (128). Furthermore, AMPA antagonists are potent anticonvulsants and are neuroprotective in case of SE (123) (123) (165).

In this preclinical research project, we will use glutamate receptor antagonists that are currently used in patients for indications other than SE. For example, Amantadine is an antiviral drug routinely used in humans for the treatment of Parkinson's disease (166). Amantadine also inhibit the NMDA receptors by accelerating the channel closure during the channel block (167). Amantadine has been found to improve cognitive outcomes and neuronal survival after traumatic brain injury in rats via blocking glutamate-induced excitotoxicity (168). Furthermore, amantadine was found to be effective as adjuvant therapy for refractory absence epilepsy (169) (170) (171).

Perampanel is a novel antiepileptic drug that has affinity for excitatory post-synaptic AMPA receptors where it acts as a selective non-competitive antagonist (172). Perampanel is the first-in-class antiepileptic agent approved by the FDA for use as monotherapy for partial seizures, and as adjunctive therapy for primarily generalized tonic–clonic seizures (173) (174). It has a long half-life that ranges between 52-129 hours, and a 100% bioavailability after an oral dose (175). The long half-life makes it an ideal choice for an individual who may have issues with adherence. Among the antiepileptics, the drug is generally well tolerated, with dizziness and somnolence being the most common adverse events experienced by users (176). We intend to evaluate the efficacy of perampanel and amantadine in a pilocarpine rat model of SE. The effect of these compounds on learning and memory performance and neuroprotection will be assessed.

2. Hypothesis and objectives:

The primary hypothesis driving the proposed research is that glutamate receptor antagonists might be effective in the treatment of SE.

Hypothesis: ***Glutamate receptor blocking agents can prevent neuronal death following SE. In addition, these agents can also preserve cognitive function.***

We will test our hypothesis according to the following objectives:

Objective 1:

To determine whether perampanel, amantadine can prevent cell death in a primary hippocampal in vitro model of glutamate excitotoxicity.

Objective 2:

To determine whether perampanel, amantadine can suppress early and late seizure activity in a pilocarpine rat model of SE.

Objective 3:

To determine whether perampanel, amantadine can prevent the development of acute and long-term memory impairment after SE.

Objective 4:

To determine whether perampanel, amantadine can prevent the development of spontaneous recurrent seizures.

Objective 5:

To determine whether perampanel, amantadine can prevent SE induced neuronal cell loss.

3. Materials and Methods:

3.1 In vitro experiments

3.1.1. Rat primary hippocampal culture

Hippocampal neuronal cultures were prepared from E18 fetuses of Sprague-Dawley rats (Charles River Canada, Montreal, PQ, Canada) as described before with some modifications (177). In brief, the hippocampus was dissected in Ca^{2+}- and Mg^{2+}-free HBSS supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml). The hippocampal tissues were then digested at 37°C with 1% trypsin-EDTA for 15 min. The reaction was quenched with fetal bovine serum (FBS) and tissues were rinsed with HBSS to remove FBS. The dissociated cell suspension was centrifugated at 800 g for 5 min; the supernatant was removed, and pelleted cells were resuspended in a chemically defined serum-free Neurobasal medium supplemented with 0.5% sodium pyruvate, 2% B27, 0.1% L-glutamine, 0.5% HEPES, 10 U/ml penicillin, and 10 µg/ml streptomycin. Cells were plated on Poly-D-Lysine coated coverslips or plates, and grown at 37°C with 5% CO_2-humidified atmosphere. Culture media were changed every 3 to 4 days. Ninety-five percentage of cultured cells were MAP2 reactive cells in this culture condition.

3.1.2. Induction of Excitotoxicity

Excitotoxicity was induced to the hippocampal neurons at DIV 12-14 using AMPA, NMDA or glutamate. All antagonists including the experimental glutamate antagonist, perampanel (10 µM) and amantadine (100 µM) were added for a period of 30 min before the agonists. Neurons were exposed to AMPA (100 µm), under non-desensitizing conditions, in the presence of cyclothiazide (CYZ) 30 µM (a preferential blocker of AMPA receptor desensitization) and the non-competitive NMDA antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-

5,10-imine hydrogen maleate (MK-801)10 µm, for 1 hour in supplemented Neurobasal medium (178). Cells were then washed with pre-warmed media and incubated for 24 h in supplemented neurobasal medium. In another set, neurons were exposed to NMDA (100 µm) and glycine (10 µm) in Mg^{2+}-free medium with NBQX (50 µm), an AMPA- receptor antagonist, added 30 min before NMDA. All drugs were applied directly to the medium and neurons were maintained at 37 °C with 5% CO_2 for further experiments. Doses of perampanel and amantadine were chosen based on dose response curve. Doses of NBQX, MK801, Cyclothiazide and glycine was chosen according to previously published papers.

3.1.3. Lactate dehydrogenase (LDH) activity

LDH is a cytoplasmic enzyme that catalyzes the interconversion of lactate and pyruvate with concomitant interconversion of nicotinamide adenine dinucleotide (NAD) and NADH (the reduced form). LDH is released from cells into the culture medium when the plasma membrane integrity is compromised. Therefore, the amount of released LDH represents the degree of cell death. The extracellular LDH level was measured using an LDH assay kit obtained from Sigma-Aldrich. The intensity of color developed was measured using spectrophotometric microplate reader at 490 nm wavelength.

3.1.4. 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT) assay

In live cells, mitochondrial enzymes have the capacity to reduce the tetrazolium dye MTT into its insoluble formazan which presents a purple color after being dissolved in acidic solvent. The MTT assay is for assessing the integrity of mitochondrial enzymes and may, under defined conditions, reflect the number of viable cells in cultures. Following the induction of excitotoxicity,

cells were incubated with MTT (0.5 mg/ml) for 3 h at 37ºC. The intensity of purple color developed was measured at 570 nm wavelength using spectrophotometric microplate reader.

3.1.5. Immunofluorescence and Hoechst staining

Neuronal cell death by apoptotic pathway was assessed by using the fluorescent Hoechst 33258 staining (179). Briefly, neurons grown on coverslips were incubated with primary mouse anti-microtubule-associated protein 2 (MAP2) antibody overnight at 4°C. After being washed three times with PBS, neurons were incubated with goat anti-mouse Alexa Flour 488 conjugate secondary antibody for one hour and then incubated with 5 μg/ml Hoechst 33342 at room temperature for 10 min. The coverslips were mounted onto slides in fluorescent mounting medium and observed under a confocal laser scanning microscope (Olympus Fluoview). The percentage of cells showing chromatin condensation (fragmented nuclei) to evenly stained nuclei was calculated by counting nuclei in six randomly selected fields per coverslip in each experimental condition. Data were obtained from three coverslips in each experimental condition with total four independent experiments.

3.1.6. Western blot and ELISA

Cultured neuronal cells or hippocampal tissues were homogenized in lysis buffer (25 mM Tris, 150 mM NaCl, 0.1% sodium deodecyl sulphate, 0.5% sodium deoxycholate and 1% Triton X-100, pH 7–8). The homogenates were kept on ice for 15 min and centrifuged at 15,000 rcf for 10 min at 4ºC. The supernatant was collected and used for western blot. Samples consisting of the same amount of total proteins were separated on 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with 5% fat-free milk for 1 h at room

temperature to block nonspecific background. The target proteins were immunoblotted with primary antibodies against caspase-3 (cell signaling, cat# 9662, 1:1000), Bcl-2 (Abcam cat# ab59348, 1:1000) and Bax (abcam ab104156, 1:1000) overnight at 4°C and then with corresponding HRP-conjugated secondary antibody for 1 h at room temperature. Membranes were reprobed with Actin (Santa cruz sc12, 1:200) on the same blot to verify consistency of protein loading. Protein bands of interest were analyzed using NIH ImageJ software and data were expressed as the percentage of the intensity of target protein to that of corresponding Actin or Histone controls. Total caspase 3 and activated caspase 3 activity were measured using RayBio® ELISA kit method (cat# PTE-CASP-D175-T). Data were presented as ratios of the activated caspase 3 to total caspase 3 within each treatment group.

3.1.7. Isolation of nuclear enriched fraction

Primary neuronal cultures or freshly dissected hippocampal tissues were homogenized using buffer A (10 mM HEPES-KOH, 10 mM KCl, 10 mM EDTA, 1.5 mM $MgCl_2$, 0.2% BSA, 1 mM DTT, 0.4% NP40 and protease inhibitors). The homogenate was incubated for 15 min on ice and then centrifuged at 850 g for 10 min at 4°C. The supernatant was saved as suspension 1. Buffer A was added to the pellet again and the suspension was centrifuged at 15,000 g for 3 min at 4°C. The supernatant was added to suspension 1 and centrifuged at 15,000g for 15 min at 4°C to remove any nuclei contaminations. The resulting supernatant was stored as cytosolic fraction. The pellets obtained from two spins were suspended in buffer B (20 mM HEPES-KOH, 400 mM NaCl, 10% glycerol, 1 mM DTT and protease inhibitors). The suspensions were incubated for 2 h on ice with occasional shaking, and then centrifuged at 15,000 g for 5 min at 4°C. The resulting supernatants (the nuclear enriched fractions) were collected and applied to western blot. The relative purity of

subcellular fractionation was assessed by blotting with subcellular markers, e.g. Histone H2A for nucleus (Abcam cat# 18255, 1:1000), 14-3-3 for cytoplasmic (Abcam cat# 9063, 1:1000), insulin receptor for cell membrane (Santa Cruz 1:200), Stearoyl-CoA desaturase-1 (SCD1) for endoplasmic reticulum (Cell Signaling cat# 2438S, 1:1000), Cytochrome C for mitochondria (Santa Cruz cat# sc-13156, 1:200), and as well as nuclear death markers, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling cat#, 1:5000) , Phosphatase and tensin homolog (PTEN) (Santa Cruz cat#, 1:500), p53 (Abcam cat# 187820) and sterol regulatory element-binding protein 1 (SREBP-1) (Novus Biological cat# NB 100-2215, 1:500).

3.2 In vivo experiments

3.2.1. Animals

Adult male Sprague-Dawley (SD) rats weighing 250-400 g were used in the study. Animal care protocols and guidelines were approved by the University of Saskatchewan Animal Research Ethics Board, following the Canadian Council on Animal Care. Rats were group housed (2 per cage) in standard polypropylene cages in a temperature controlled (21°C) colony room on a 12/12 h light/dark cycle. Experimental procedures were carried out during the light phase. Rats were divided into 6 groups:

Group 1: Vehicle control (n=24).

Group 2: Perampanel control (n=8).

Group 3: Amantadine control (n=8).

Group 4: Pilocarpine + Vehicle (n=24).

Group 5: Pilocarpine + Perampanel (n=38).

Group 6: Pilocarpine + Amantadine (n=38).

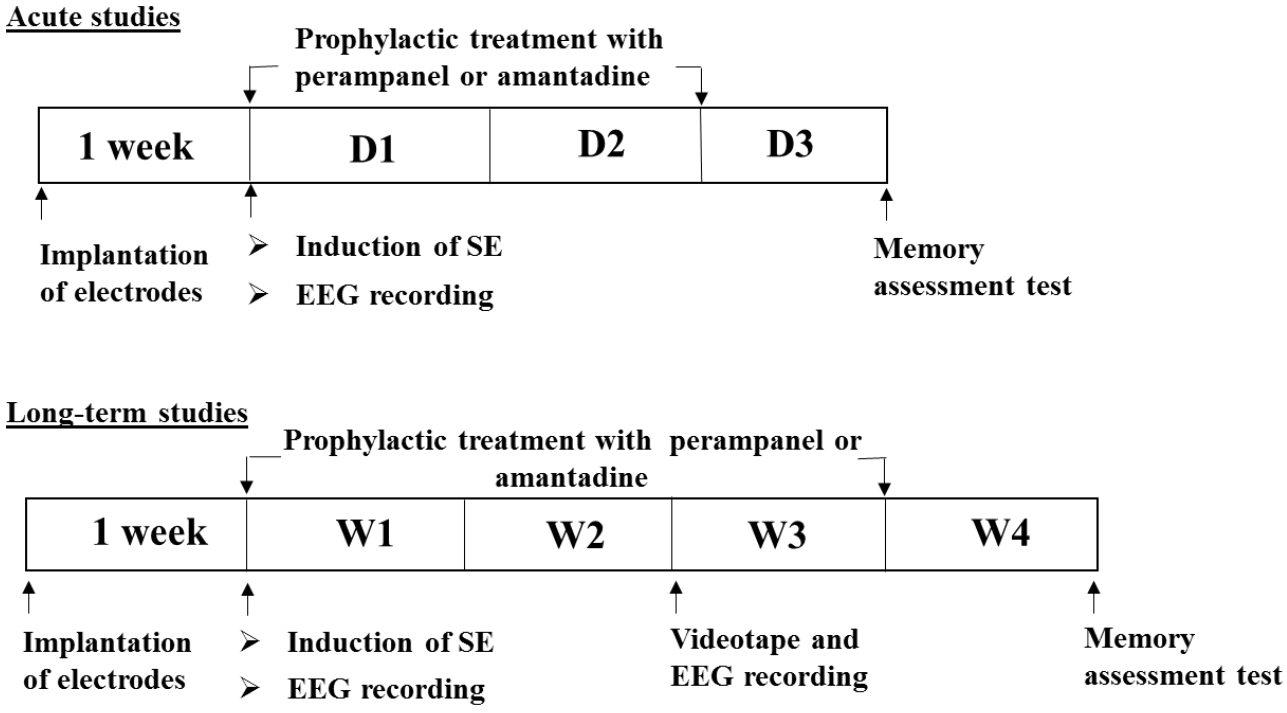


Fig.1. Schematic illustration of the protocol to investigate the effects of perampanel and amantadine in a pilocarpine induced rat model of Status Epilepticus. Status epilepticus was induced by scopolamine methyl bromide, followed by injection of a high dose of pilocarpine. Drugs or vehicle were injected 10 or 60 min after seizure onset. Animals were planted with electrodes 1 week prior to SE induction. Rats were tested with Y maze and NOR 72 hours or 1 month after SE onset, and then sacrificed to assess neuronal injury. In long term study, rats from different treatment groups were monitored for the detection of spontaneous recurrent seizures 2 weeks after SE induction.

3.2.2. Electrode implantation and Electroencephalography (EEG)

All surgeries were performed as described before with some modifications (180). The animal was anesthetized using 5% isoflurane and positioned in a Kopf stereotaxic instrument. Anesthesia was maintained throughout the surgery with isoflurane gas (2% isoflurane delivered in O_2). The incisor bar was adjusted until bregma was leveled with lambda.

One unipolar stainless-steel depth electrodes (E363-1-SPC stainless steel electrode, bare diameter 0.25 mm, insulated diameter 0.28 mm, Plastics One, Roanoke, VA) was introduced into the brain parenchyma to record intrahippocampal EEG activity. The stereotaxic coordinates relative to bregma according to the atlas of Paxinos and Watson (2007) were (Anterior Posterior (AP)=–3.3mm, Medial Lateral (ML)=2mm and Distal Ventral (DV)=–3.4 mm). Another unipolar electrode was implanted into the cortex (AP + 0.5 mm, ML 4.0, DV − 1.2). A third depth electrode was positioned in the white matter of the cerebellum (AP=–11mm, ML=5.3mm, DV=–5.6mm) to serve as the reference. A fourth screw electrode was positioned in the occipital bone to serve as the ground. The other end of the electrodes was inserted into a plastic pedestal (Plastics One) and the entire setup was secured by acrylic adhesive. The wound was closed with surgical sutures and Anafen was given on the surgery site for postoperative analgesia as follows: one dose 30 min prior to surgery (5 mg/kg s.c.) The same dose was repeated for 3 days after surgery. Animals were allowed to recover for a period of 1 week.

3.2.3. Induction of seizure

Animals were injected with methyl-scopolamine (1 mg/kg, s.c) 15 min prior to pilocarpine injection to minimize peripheral cholinergic effects. Pilocarpine (380 mg/kg, i.p) was dissolved freshly in 0.9% saline. The beginning of status epilepticus (SE) was considered when the animal

suffered a stage 4-5 motor seizure in Racine's scale and high frequency spikes on EEG. SE was terminated one hour after the development of SE by using pentobarbital injection (25 mg/kg i.p. for rats in group II and IV). Seizures were monitored for five hours by recording EEG. Perampanel (8 mg/kg i.p-dissolved fresh in 1:1:1 (v/v) distilled water, dimethyl sulfoxide, and polyethylene glycol 300) or amantadine (45 mg/kg i.p-dissolved fresh in saline) was administered 10 or 60 min after the onset of seizure. Since perampanel has longer half-life, the drug was given in tapering down plan (4 mg/kg for week I, 2 mg/kg for week II and 0.5 mg/kg for week III) whilst amantadine was given twice daily and stopped a week before the behavioral assessment. Two hours after pentobarbital injection, rats were given subcutaneous injections of 5% dextrose and 0.9% saline (2 ml/rat) for hydration and were monitored daily for adequate food and water intake by measuring body weight.

3.2.4. Assessment of Behavioral seizures

Following pilocarpine injection, the animals were observed for seizure scoring according to Racine criteria with slight modification (181). The seizure scoring was as follows: Stage 1, immobilization, eye blinking, twitching of vibrissae and mouth movements; Stage 2, head nodding, often accompanied by severe facial clonus, piloerection; Stage 3, straub tail, forelimb clonus; Stage 4, rearing; Stage 5, rearing, falling and generalized convulsions.

3.2.5. Spatial memory test

Y-maze apparatus with three enclosed arms (60 cm length × 16 cm width × 30 cm height) was used for spatial memory as described previously (182). Visual cues outside but around the maze were used to assess hippocampal-dependent spatial recognition memory. The test consisted of two

trials with a ninety minutes interval in between. Rats were transported to the behavioral testing room in their home cages at least 1 h before testing. In the first training (acquisition) trial, rats were placed in the maze facing the end of a randomly chosen arm (start arm) and allowed to explore the maze for 15 min with one arm closed (novel arm). Rats were returned to their home cages until the second (retrieval) trial, during which they could explore freely all three arms of the maze. The time spent in each arm was measured using video recordings. Rats entering an arm with all four paws was counted as an entry. Data were presented as the percentage of the time spent in the novel arm to the total time in all three arms during the 5-min retrieval trial. The maze was cleaned with 40% ethanol between trials to ensure that animal's behavior was not guided by odor cues.

3.2.6. Novel Object Recognition test

The novel object recognition task was used to evaluate recognition memory (183). This task consisted of two phases, a learning phase and a memory phase. During the learning phase, rats were placed into the behavioral arena for a period of 15 min and allowed to explore two identical stimulus objects before being placed back into the home cage. After a ninety-minute delay, rats were placed back into the arena where one of the two identical objects were replaced by an entirely new stimulus object. The recognition index (RI, representing the time spent investigating the novel object (T_{novel}) relative to the total object investigation) was used as the main index of retention, which was calculated according to the following formula: $RI = T_{novel} / (T_{novel} + T_{familiar})$. The arena and objects were cleaned with 40% ethanol between the trials to prevent the existence of olfactory cues.

3.2.7. Recording for Spontaneous Recurrent Seizures (SRSs)

Arida et al (1999) and Hoexter et al (2005) previously demonstrated that the average latency onset to SRSs in rats treated with the pilocarpine protocol was 11-18 days (59) (184). In the present study, rats were observed for behavioral and electrographic seizure for 8-12 hour / day for 2 weeks starting three weeks after the induction of seizure. Because the frequency of SRSs in rats after pilocarpine-induced SE is much higher during the light (diurnal) compared to the dark (nocturnal) period (62) (63), all recordings for spontaneous seizures were done during the light period (7 a.m. – 6 p.m.). Electrographic seizures were analyzed offline and seizure was confirmed by manual review of the tracing morphology of EEG recording and of the taped videos. Since most SRSs following pilocarpine-induced SE are generalized (185), only the occurrence of class 4/5 behavioral seizures was included in the spontaneous seizure analysis. A rat was considered epileptic after exhibiting one or more SRSs. Outcome measures were the percentage of animals that developed SRS and the number of SRS recorded per week.

3.2.8. Fluoro-Jade C staining (FJC)

To examine the degree of dying neurons in brains of above animal models, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 1× PBS, followed by phosphate buffered (pH 7.4) 4% paraformaldehyde (PFA). The brains were removed immediately and immersed in 4% PFA for another 24 hours. The brain samples were then cut into 30 μm thick sections using a vibratome. To visualize the degenerative neurons, FJC staining was carried out in the following standard procedures: (1) pre-incubation with alcohol-sodium hydroxide mixture, the sections were immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min, followed by 70% alcohol and distilled water each for 2 min; (2) pre-stained

with potassium permanganate, the sections were then transferred into a solution of 0.06% potassium permanganate for 10 min, and rinsed in distilled water for 2 min; (3) FJC staining, the sections were immersed into 0.0001% solution of FJC dye dissolved in 0.1% acetic acid vehicle and stained for 10 min; (4) post-staining with distilled water wash, after incubation in the FJC working solution, the slides were washed three times in distilled water each for 1 min and left to dry overnight in darkness at room temperature; (5) Examination under fluorescent microscope, sections were air-dried, cleared in xylene for at least 1 min and then cover slipped with DPX. Finally, FJC-stained brain sections were examined under a fluorescence microscope (Olympus, BX-60). The FJC-positive staining structures exhibited bright green color. Four sections were used from each brain from 6 rats n=6. The fluorescent cells were counted using Image J software.

3.2.9. NeuN and GFAP immunohistochemistry

Immunohistochemistry on PFA fixed free-floating sections was performed on brains sectioned at a thickness of 30 µm. Briefly, the sections were treated with 0.1 M Tris buffer (TB) containing 1% hydrogen peroxide for 30 minutes. The slices were washed in phosphate-buffered saline solution (PBS 0.1 M, pH 7.4) containing 0.1% Triton X-100. Then incubated in blocking solution (0.5% Triton X-100, 10% bovine serum albumin for 1 hour. Sections were incubated overnight at 4°C in the primary antibody diluted in 0.1% Triton X-100, 2% bovine serum albumin. The antibodies used were as follows: rabbit anti-glial fibrillary acidic protein (GFAP) (1:200, Thermofisher) and mouse anti-neuron-specific nuclear protein (NeuN) (1:500, Chemicon). Biotinylated secondary antibodies (goat anti-rabbit, goat anti-mouse, all from Vector Laboratories, Burlingame, CA), diluted at 1:200 for 2 hours, followed by standard avidin-biotin complex (ABC; Vector). The tissue-bound peroxidase was then developed using 3,3-diaminobenzidine (DAB)

visualization procedure (1-3 min). The sections were mounted on slides, and cover slipped with DPX. The same brain sections of CA1, CA3 and DG regions were imaged using a magnification of 400x.The numbers of positive cells were counted. The data were presented as mean and standard deviation. All measurements were repeated three times and the mean value was used.

3.3 Statistical analyses

Significance was set at $p < 0.05$ and assessed by one-way or two-way ANOVA with post hoc analyses relying on Tukey's test (GraphPad Prism v5.0). Data are represented as mean ± SD (standard deviation).

4. Results:

4.1. In vitro results:

4.1.1. Perampanel and amantadine reversed cytotoxicity induced by AMPA or NMDA, respectively, in primarily cultured hippocampal neurons.

Changes in neuronal viability associated with the stimulation of AMPA receptors under desensitizing or non-desensitizing conditions were analyzed by the MTT assay (Fig. 2a) and the release of LDH (Fig. 2b). Activation of AMPA receptors under non-desensitizing conditions in the presence of AMPA (100 μM) plus CYZ (30 μM), was associated with a significant decrease in cell viability (by about 45%; $P<0.001$). Substantial prevention of cell death induced by AMPA in the presence of CYZ was observed after incubation with Perampanel as evidenced by increased percentage of live cells ($P < 0.001$) and decreased ($P < 0.01$) LDH levels in media (Figs 2a and b). In order to evaluate the role of NMDA receptor activation in hippocampal neuronal death, we examined the effect of NMDA receptor stimulation upon exposure to NMDA (100 μM) plus glycine (10 μM) in Mg^{2+}-free medium. The medium contained NBQX to eliminate the effect of AMPA receptors. Under these conditions, the percentage of viable cells were greatly decreased ($P < 0.01$), and increased the release of LDH ($P < 0.05$). Furthermore, specific blockade of NMDA receptors with the experimental drug (amantadine) enhanced the percentage of viable cells ($P < 0.05$) and decreased LDH release ($P < 0.05$) in media compared to the NMDA exposed cells.

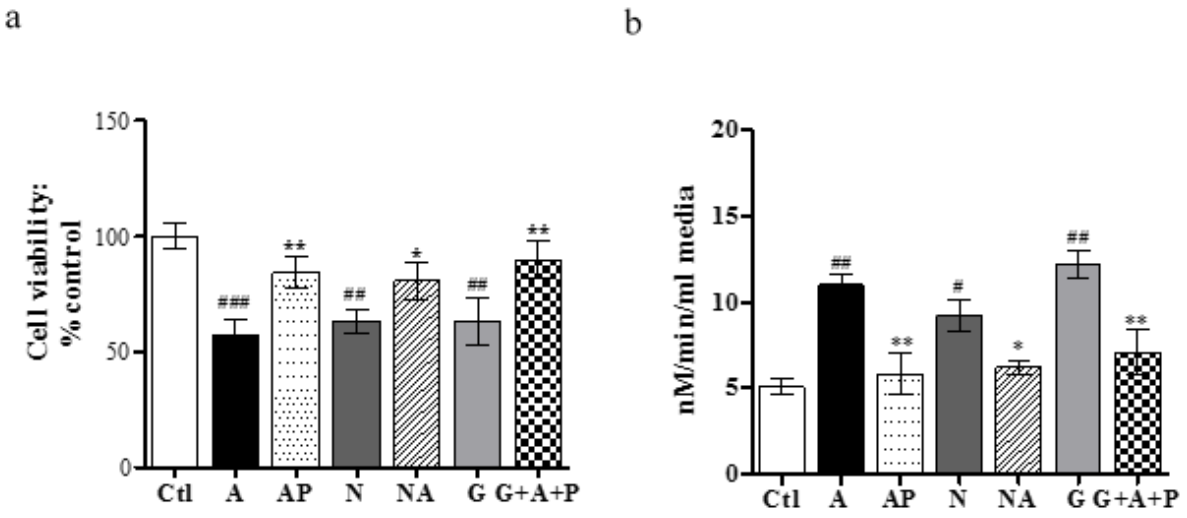


Fig.2. Effect of perampanel or amantadine on the viability of primary hippocampal neurons. Cell viability was assessed by (a) MTT assay and (b) LDH assay. Results were expressed as percentage live cells for MTT and enzyme activity (nM/min/ml media) for the LDH assay. Group abbreviations (Ctl= Control, A= AMPA, AP= AMPA+Perampanel, N= NMDA, NA= NMDA+amantadine, G= Glutamate, G+A+P= Glutamate+Amantadine+Perampanel). Values were expressed in mean ± SD. Data were obtained from five independent experiments with triplicates in each. ##, ###, $p < 0.01$ and 0.001 vs control respectively; *, **, $p < 0.05$ and 0.01 vs the corresponding agonist stimulus groups.

4.1.2. Perampanel and amantadine reduced apoptosis induced by AMPA or NMDA, respectively, in primarily cultured hippocampal neurons.

Features of apoptotic cells were examined by counting the number of hippocampal cell nuclei labelled with the fluorescent probe Hoechst, a method used to analyze chromatin condensation and/or fragmentation. Compared with the control cells, selective activation of AMPA receptors under non-desensitizing conditions induced by CYZ, and in the presence of MK-801 increased the number of apoptotic cells. Furthermore, hippocampal neuronal death was attenuated upon blockade of AMPA receptors in the presence of perampanel. NMDA exposed cultures in the presence of glycine and the AMPA antagonist (NBQX) showed high number of apoptotic nuclei, which was significantly attenuated by amantadine. The number of neurons undergoing apoptosis was almost completely prevented upon blockade of both NMDA and AMPA receptors, respectively, in the presence of perampanel and Amantadine (Fig. 3).

Features of apoptosis were also examined by measuring caspase 3 activation using western blot and caspase-3 ELISA. Neurons treated with AMPA, NMDA or glutamate showed increased caspase 3 activation when compared to normal control cells. Treatments with perampanel ($P < 0.001$), amantadine ($P < 0.01$), and their combination ($P < 0.001$) significantly decreased the level of caspase-3 activation when compared to AMPA, NMDA and glutamate alone, respectively, which were consistence with the ELISA results (Fig. 3c).

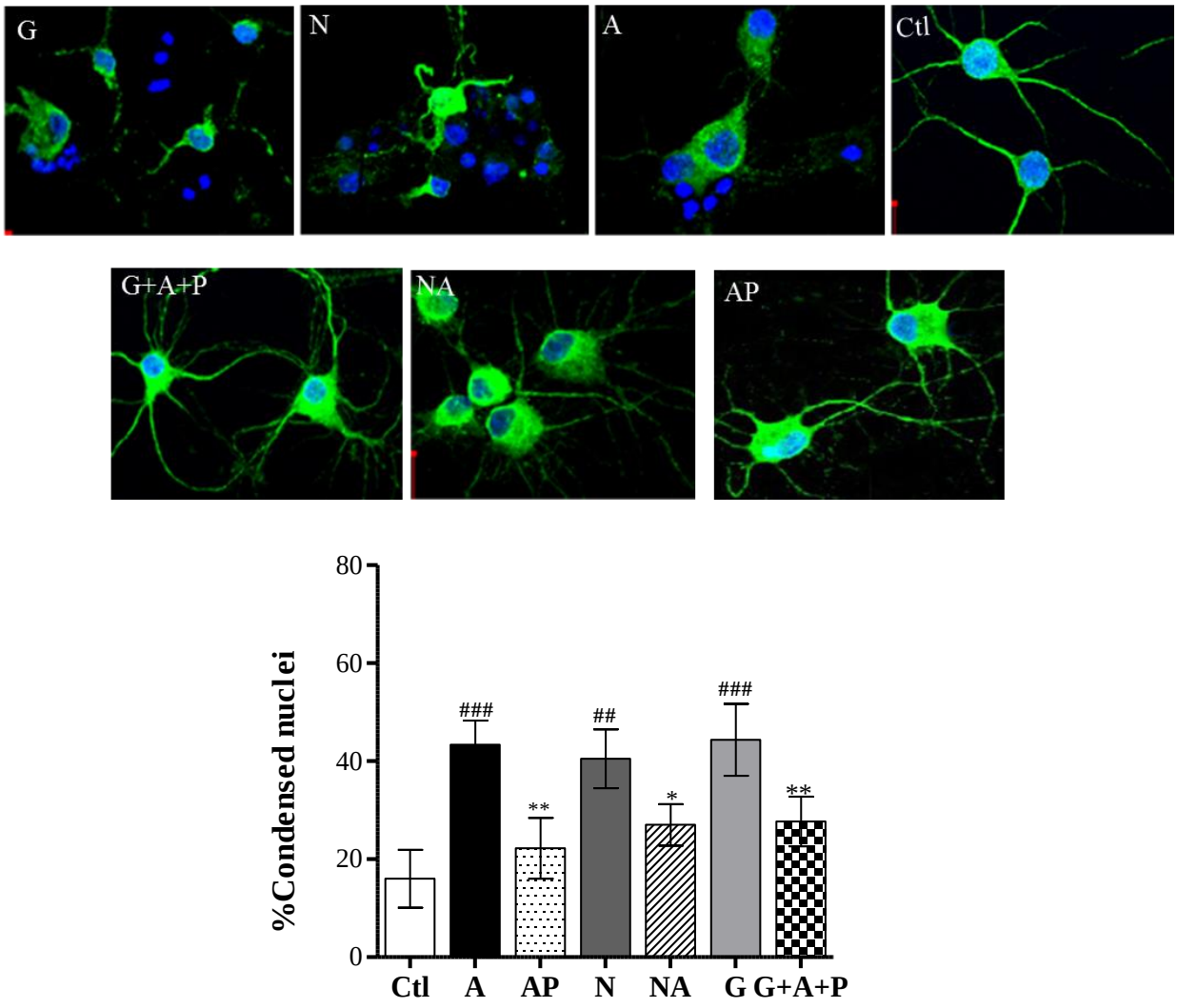


Fig.3. Effect of perampanel or amantadine on hippocampal neuronal death in glutamate induced excitotoxicity. Representative images of primary hippocampal cells stained with MAP2 (Green) as a neuronal marker and Hoechst 33258 as a nuclear marker. Total cells and cells with condensed /fragmented nuclei were counted in six random fields from each coverslip. The results represent the mean ± SD from four independent experiments and were expressed as percentage of condensed nuclei. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. ##, ###, $P < 0.01$ and 0.001 vs control respectively; *, **, $P < 0.05$ and 0.01 vs the corresponding agonist stimulus group.

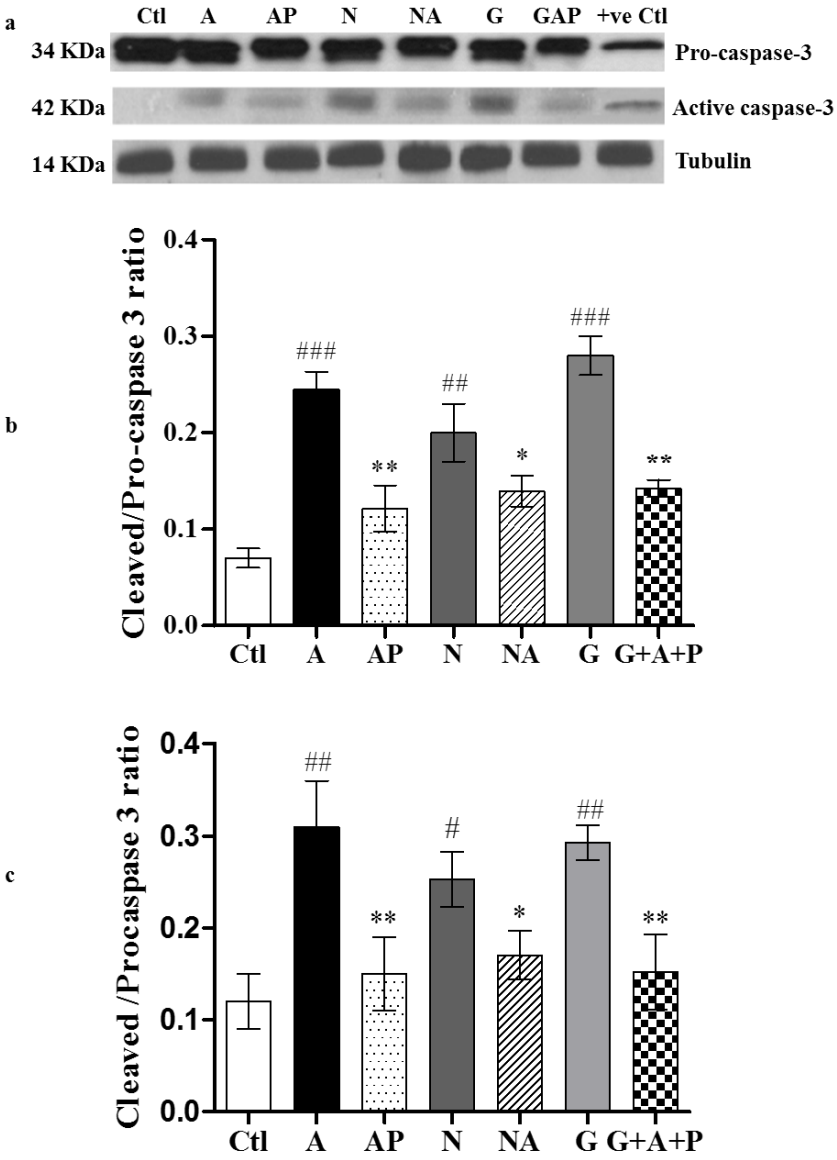
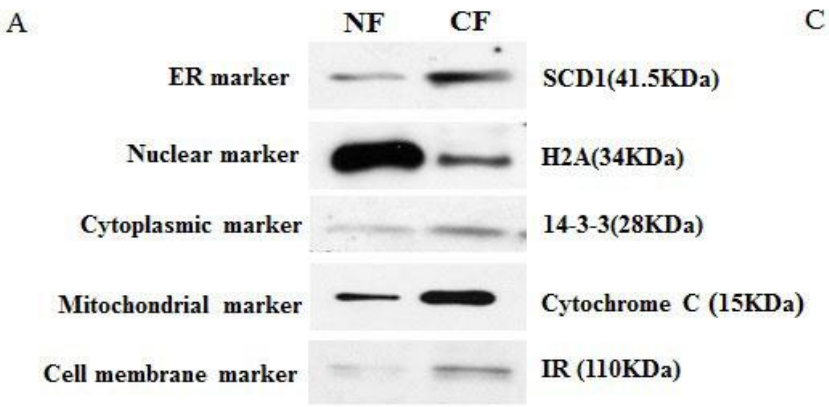


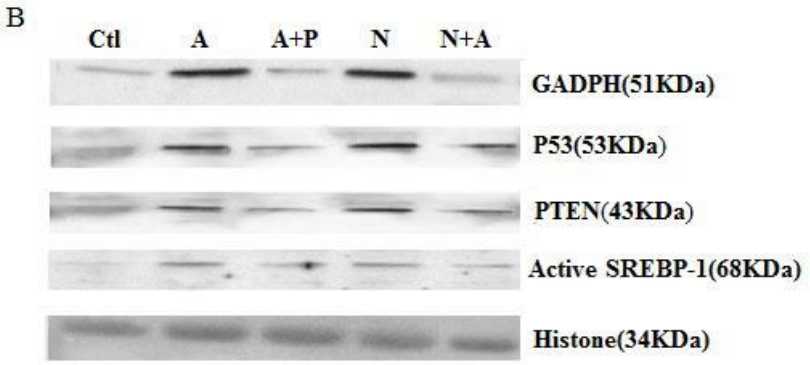
Fig.4. Effect of perampanel or amantadine on caspase 3 activation induced by glutamate excitotoxity in hippocampal neuronal culture. (a) Representative western blot for caspase 3 from three independent experiments and histogram, (b) graph representing the percentage of active versus total caspase 3 ratio as normalized to the loading control. (c) graph representing active/pro-caspase-3 ratio using ELISA technique. Values were expressed in mean ± SD. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. ###, $p < 0.001$ vs control; **, ***, $p < 0.01$ and 0.001, vs respective agonist group.

4.1.3. Perampanel and amantadine inhibited the AMPA and NMDA induced expression of nuclear death markers in the nucleus.

Analysis of subcellular markers: The nuclear enriched fractions and cytosolic fractions of neuronal homogenate were blotted with various subcellular protein markers like histone, 14-3-3, cytochrome C, SCD1 and insulin receptor and it was observed that nuclear enriched fraction prepared in this experiment was selectively higher in histone confirming nuclear enrichment with little subcellular contaminations (Fig.5a). Similar data were observed in three separate experiments.

Nuclear enriched fractions were isolated from hippocampal cell cultures and studied for cell death signals. Cells treated by AMPA, NMDA showed increased expression of apoptotic markers like p53 ($P<0.01$, $P<0.05$ for AMPA and NMDA treated cells respectively), GAPDH ($P<0.01$, $P<0.01$ for AMPA and NMDA treated cells respectively) when compared to control levels. Perampanel or Amantadine treatment significantly reduced the levels of those markers (Fig.5b, c). PTEN and the SREBP1 expression was higher in the nuclear fractions of AMPA or NMDA treated cells but not statistically significant in comparison to the control levels due to variation in standard error values (Fig.5c)


A
NF
CF
ER marker
SCD1(41.5KDa)
Nuclear marker
H2A(34KDa)
Cytoplasmic marker
14-3-3(28KDa)
Mitochondrial marker
Cytochrome C (15KDa)
Cell membrane marker
IR (110KDa)


B
Ctl
A
A+P
N
N+A
GADPH(51KDa)
P53(53KDa)
PTEN(43KDa)
Active SREBP-1(68KDa)
Histone(34KDa)

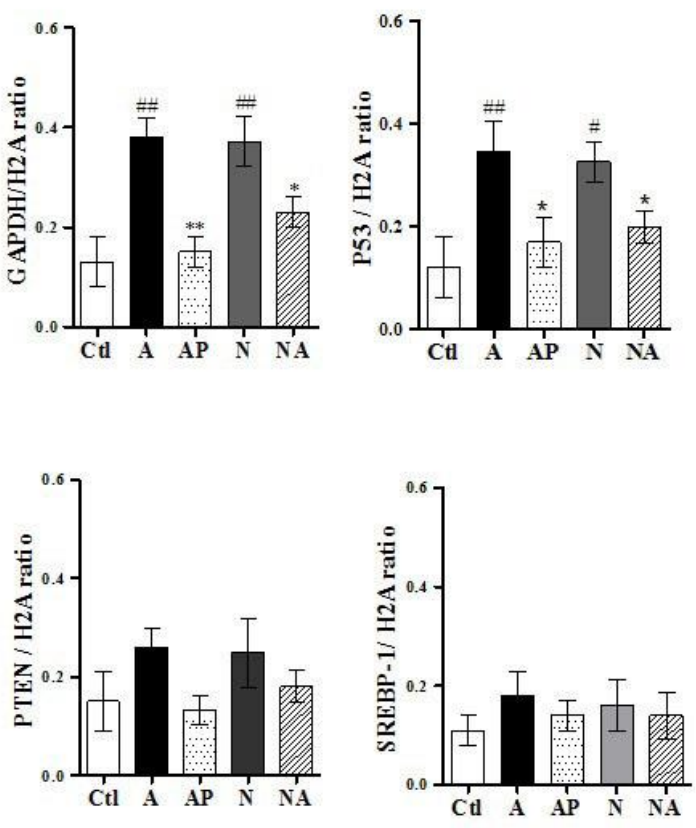

C
GAPDH/H2A ratio
P53 / H2A ratio
PTEN / H2A ratio
SREBP-1/ H2A ratio
Ctl
A
AP
N
NA

Fig. 5. Effect of perampanel or amantadine on the nuclear death markers in hippocampal neuronal cultures. (a) Representative western blot for subcellular markers indicating the absence of significant contamination in nuclear fractions (NF=nuclear fraction, CF=cytosolic fraction). (b) Western blot representative images of the following proteins (a) GAPDH (b) P53 (c) PTEN (d) SREBP-1 (c) Histone (H2A), in NF isolated from primary hippocampal cells treated with AMPA or NMDA with or without perampanel or amantadine. (c) Graphs representing the ratio of the above protein levels to H2A. Group abbreviations (Ctl= Control, A= AMPA, AP= AMPA+Perampanel, N= NMDA, NA= NMDA+amantadine). Data were obtained from three independent experiments. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. #, ##, $p<0.05$ and 0.01, respectively vs control. *, ** $p < 0.05$ and 0.01 vs the corresponding agonist stimulus groups.

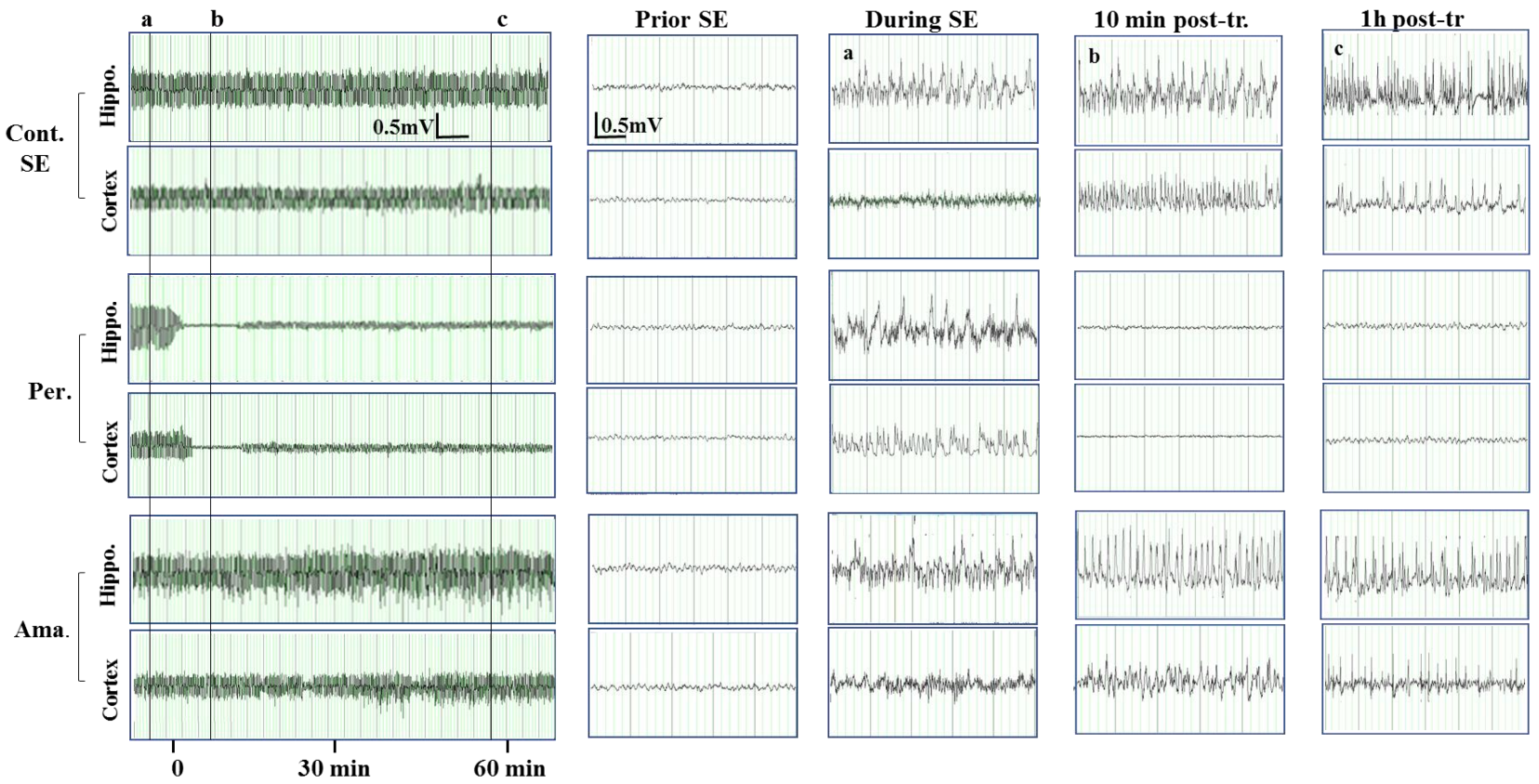
4.2. In vivo results:

4.2.1. Perampanel, not amantadine terminated pilocarpine-induced status epilepticus in rats

In order to quantitatively compare the responses to the different treatments administered after 10 min duration of status epilepticus, the latency to seizure termination was monitored from EEG recordings. Figure 6 illustrated a typical EEG recording of perampanel or amantadine treatment in which status epilepticus had been induced by i.p. injection with a 380 mg/kg dose of pilocarpine. Treatments were initiated 10 min after induction of seizure. Perampanel (8mg/kg) caused a cessation of seizure behavior rapidly with sustained suppression of electrographic seizures, as illustrated in Figure 6. The latency of seizure termination in the perampanel treated group was 8.5 ± 4.3 min. In contrast, amantadine (45mg/kg) showed no anti-seizure effect. Conversely, amantadine treated animals showed very intense behavioral seizure with hyperlocomotion, jumping, rearing and falling and more intense seizure on EEG recording. In a pilot experiment (n = 4), amantadine alone failed to stop or reduce the intensity of seizure, and rats were found dead the next day. In order to reduce the mortality rate in this group, a dose of 10 mg/kg of pentobarbital was given 2 hours after the amantadine dose was given. In a separate series of experiments, the treatments were administered 60 min after continuous electrographic seizure activity. Such late administration of perampanel still presented high efficiency in terminating electrographic status epilepticus (latency, 18.2 ± 3.6 min) with only minimal recurrence of seizure. Late administration of amantadine alone was not effective to terminate electrographic status epilepticus (latency, 126.3 ± 17.9 min); it has to be in conjunction with pentobarbital and even though there was modest recurrence of high electrographic waves in this treatment group. Overall,

when administered early or late, perampanel was more effective than amantadine in suppressing seizure activity.

a


a
b
c
Prior SE
During SE
10 min post-tr.
1h post-tr
Cont. SE
Per.
Ama.
Hippo.
Cortex
0.5mV
0.5mV
a
b
c
0
30 min
60 min

b

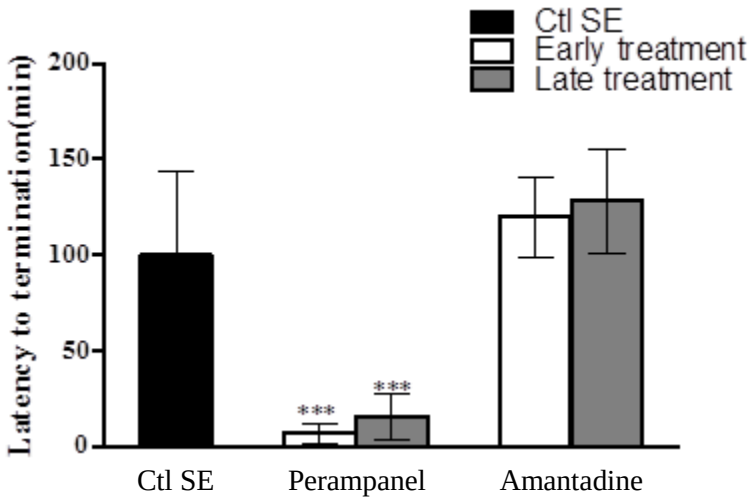
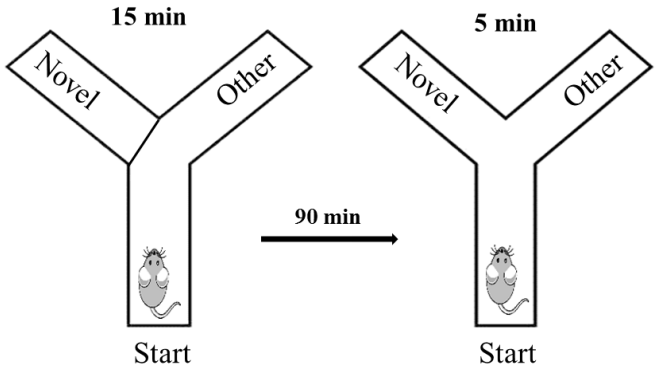


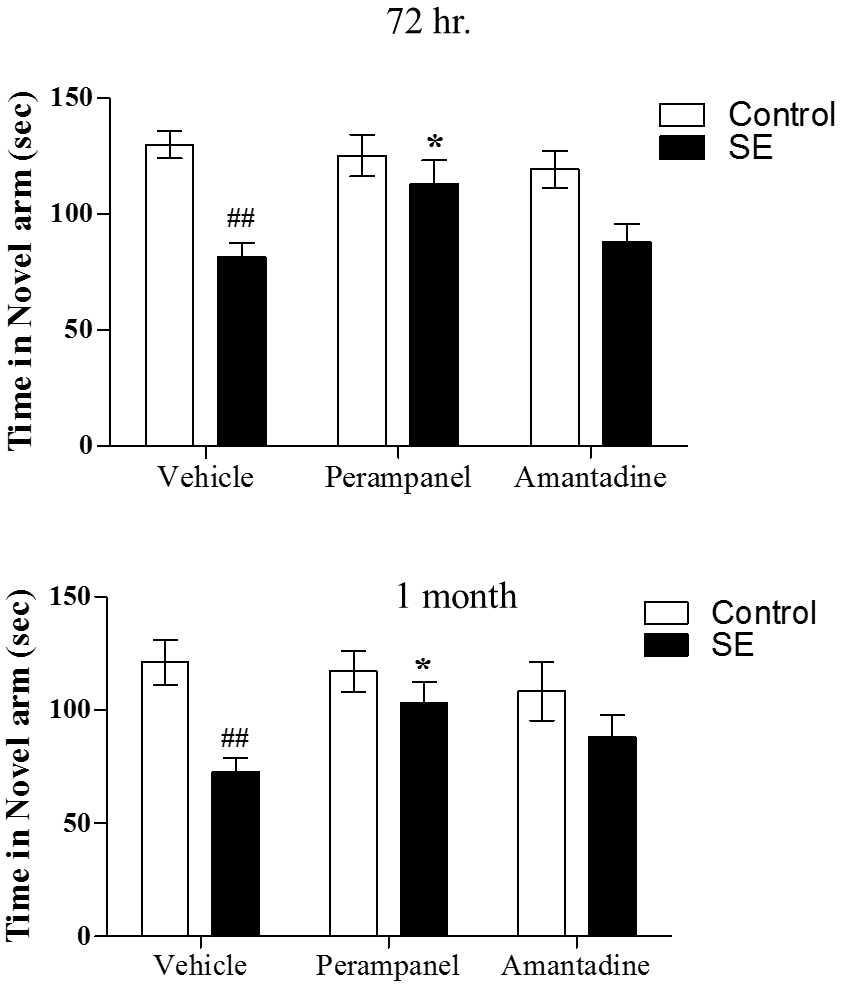
Fig. 6. Effect of perampanel or amantadine on terminating ongoing seizures. (**a**) Representative EEG recording from hippocampal and cortical electrodes. The left panels show the compressed EEG from SE, perampanel and amantadine animals up to 75 min following treatment. The right panels show the magnified 6 secs prior to SE, during SE, 10 min post-treatment and 1-hour post-SE. EEG traces prior to SE or following to SE (marked by vertical lines a-c at 0.5 mV, horizontal bar=1 sec). (**b**) graph shows the effect of early and late treatment (10 and 60 min after onset of status epilepticus) with perampanel and amantadine on the duration of EEG seizure activity. The Y axis represents the mean time to the first termination of continuous seizure activity. N= 12 animals. Values were expressed in mean ± SD. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. ***p < 0.001 indicate comparisons with SE group.

4.2.2. Perampanel, but not amantadine attenuated cognitive deficits in SE rats

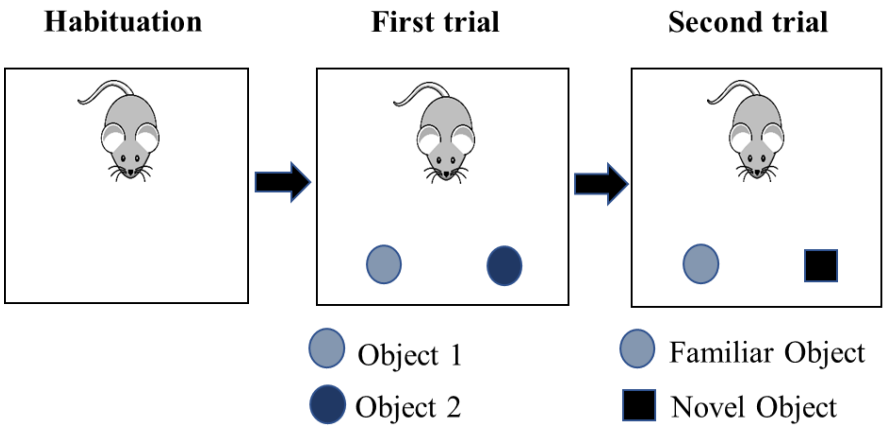
In order to examine the efficiency of perampanel or amantadine on attenuation of cognitive impairment induced by SE, Y maze and NOR were performed in rats. Figure 2. A showed that the performance in the Y maze was significantly impaired in the SE group compared to the sham control at 72 h and 1 month after SE initiation. When rats were treated with perampanel either in early (10 min post-SE) or late (60 min post-SE), the performances were improved significantly after 72 hours or 1-month treatment compared with the SE group ($p < 0.05$). On contrast, SE rats treated with amantadine did not show any improvement on exploring the novel arm in 72-hour and 1 month after SE. Similarly, in the NOR test, rats in perampanel treated group spent more time on exploring novel object than the SE rats, significantly after 1-month treatment showed in figure.7b ($p<0.05$). Again, rats with amantadine treatment did not show any improvement on NOR performance. Together, these observations indicate that perampanel attenuated both short and long-term memory deficit in pilocarpine-induced SE rats. This might be an important facet of perampanel that attributed to block the progress of SE and improve the quality of life.

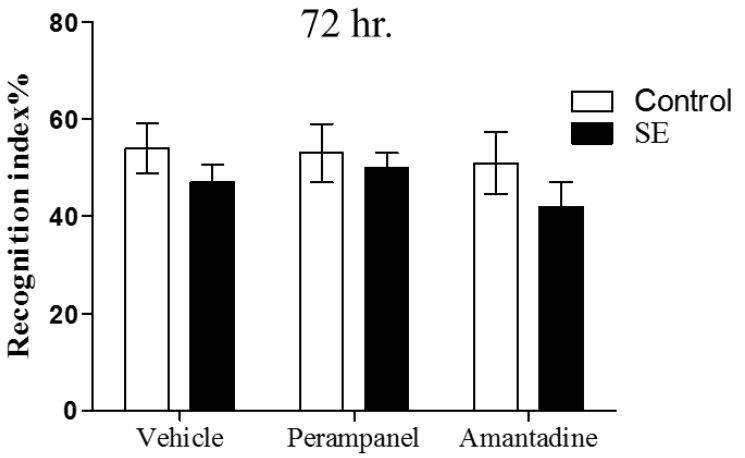
a


15 min
5 min
Novel
Other
Novel
Other
90 min
Start
Start


72 hr.
Time in Novel arm (sec)
150
100
50
0
##
*
Control
SE
Vehicle
Perampanel
Amantadine
1 month
Time in Novel arm (sec)
150
100
50
0
##
*
Control
SE
Vehicle
Perampanel
Amantadine

b


Habituation
First trial
Second trial
Object 1
Object 2
Familiar Object
Novel Object


72 hr.
Recognition index%
80
60
40
20
0
Control
SE
Vehicle
Perampanel
Amantadine

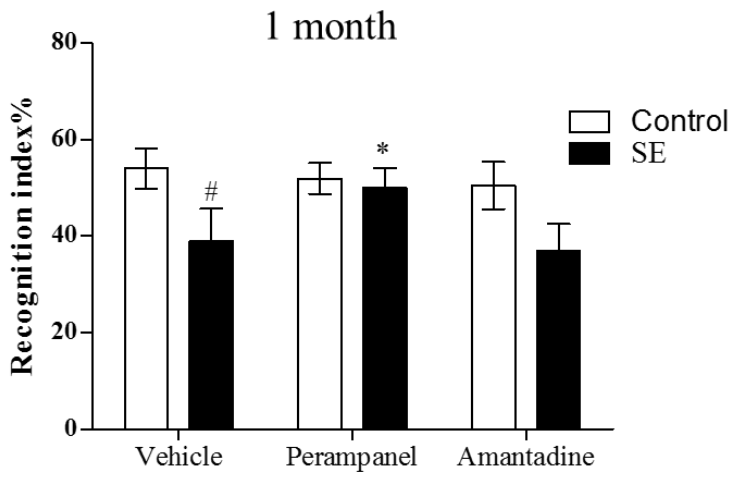

1 month
Recognition index%
80
60
40
20
0
#
*
Control
SE
Vehicle
Perampanel
Amantadine

Fig. 7. Effect of perampanel or amantadine on cognitive function in pilocarpine induced status epilepticus rat model using Y-maze and NOR. (a) Cartoon represents Y-maze (left) and graph (Right) represents the time spent in novel arm (seconds) during retrieval trial at 72 hrs and 1 month after initiation of SE. Groups include control (Ctl), pilocarpine (SE), perampanel + pilocarpine (per) and amantadine + pilocarpine (Aman.). (b) Cartoon represents NOR (left) and graph (Right) represents the recognition index of NOR test from the above-mentioned groups. Values were expressed as mean ± SD. N=12, Mean difference between the groups were analyzed using two-way ANOVA followed by Bonferroni posttest in graphpad prism 5.0. #, ## $p < 0.05$ and 0.01, respectively vs control; *, $p < 0.05$ vs seizure group.

4.2.3. Perampanel, not amantadine exerted the antiepileptogenic effect in SE rats

We then examined whether perampanel and amantadine treatment has a long-term effect on the development of SRSs. Neither of rats treated with perampanel 10 min after SE developed SRSs. However, when given 60 min after SE, 2 out of 10 rats developed SRSs after the termination of treatment. No rats experienced SRSs during perampanel dosing period.

On contrast, during the 2 weeks observation for SRSs, rats treated with either vehicle or amantadine, spontaneous seizures were noted in 64% of vehicle treated SE rats and 52% and 63% of amantadine treated rats 10 and 60 min post-SE respectively. (Fig. 3b). The first spontaneous seizure was recorded after SE induction at 17 days in the vehicle treated rats, and 19 days in the amantadine treated rats. The frequency of seizure was significantly different in the two groups in that less frequent seizure were observed in the amantadine treated groups during the 1-month study. Thus, under our experimental conditions, the prophylactic treatment with amantadine during or after SE exerted no effect on the occurrence of spontaneous seizures.

Some rats in the vehicle SE group experienced stage 5 seizure during handling and daily observation before the start of the EEG and videotape recording for SRS. It should be noted that no EEG/video recording was performed during the first 2 weeks of treatment immediately after SE, so that seizure data from this period relate to all spontaneous seizure which occurred during handling of animals. In the last 2 weeks of the study, all the seizure data were from 8-12hours EEG/video recording daily for 14 days.

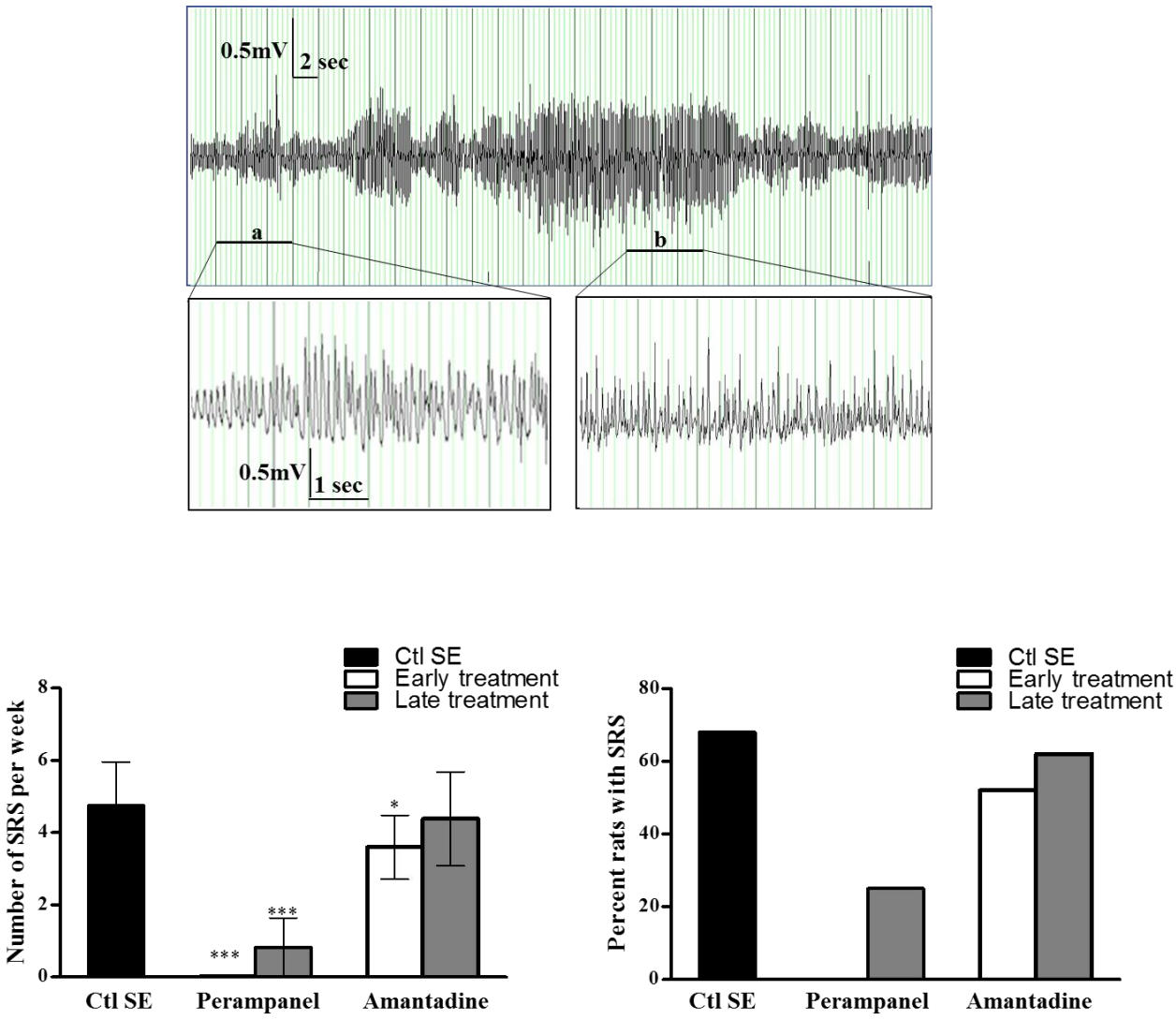
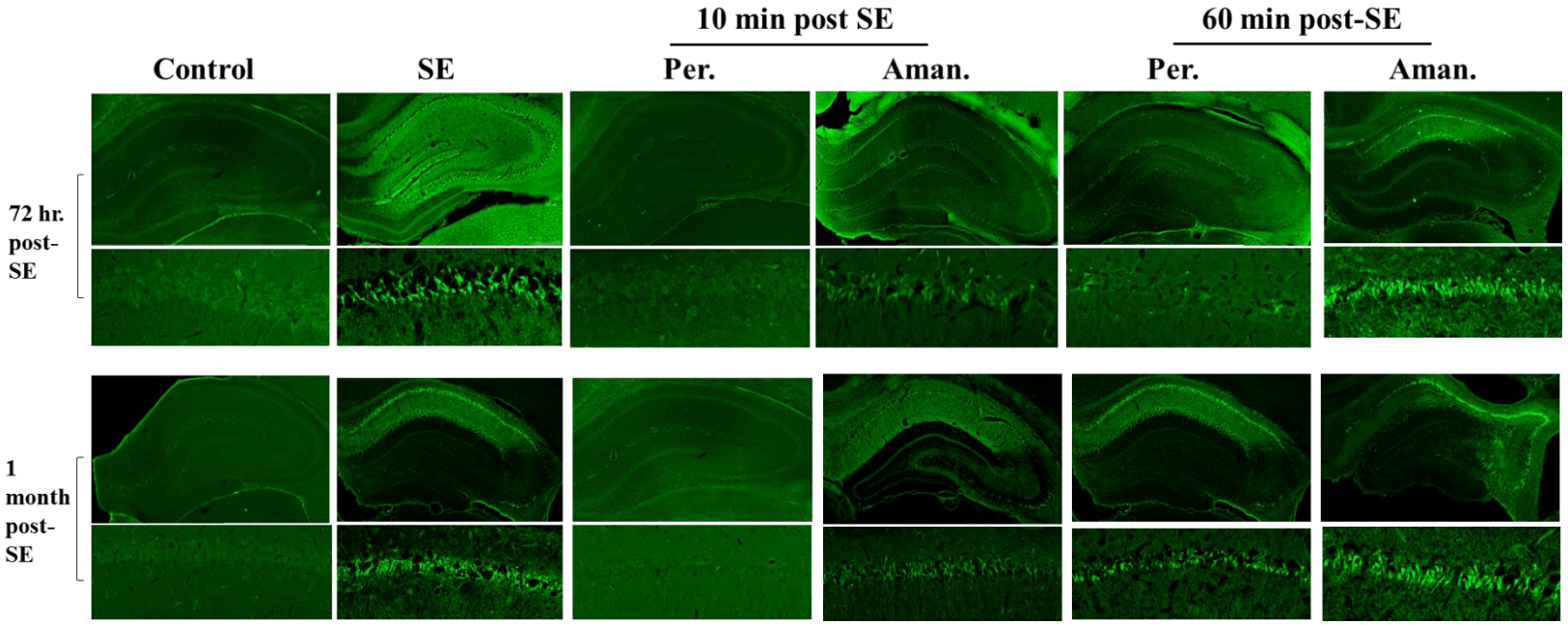


Fig.8. Effect of perampanel or amantadine on epileptogesesis in pilocarpine induced status epilepticus rat model. (**a**) The upper panel shows the compressed 120 sec. EEG of a Spontaneous Recurrent Seizures (SRSs). The lower panel shows the magnified 6 sec. EEG traces marked by horizontal lines a,b. Vertical bar = 0.5 mV, horizontal bar = 20 sec. (**b**) graph showing the number of SRSs per week. Values were expressed as mean ± SD. N=12, Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. *, *** $p < 0.005$ and 0.001 respectively versus the SE group. (**c**) the graph represents the percentage of rats developed SRSs in the different treatment groups.

4.2.4. Perampanel, not amantadine inhibited SE-induced neuronal loss in SE rats

In order to investigate the mechanism perampanel exerted on anti-epileptogenesis, brain sections in the hippocampal regions from each group were applied to FluoroJade C staining (FJC) and NeuN immunohistochemistry analysis. Visual inspection of FJC stained sections indicated severe neuronal degeneration in the CA1, CA3 and hilar regions of rat brains 72 h and 1 month after initiation of SE (Fig. 9). In addition, NeuN immunohistochemistry revealed neuronal loss in these parts of the hippocampus (Fig. 10). In contrast, there was no obvious neuronal damage in the hippocampal formation of perampanel treated rats when given 10 min after SE. This was confirmed by counting of neurons in the CA1, CA3 and hilar regions. When given 60 min after SE onset, the protective effect of perampanel was compromised only within the CA1 and hilar area in 56 % of rats. Nevertheless, no FJC positive cells were seen in the CA3 region.

Amantadine, in contrast, showed a different profile in hippocampal cell death in SE rats. When given amantadine 10 min post SE, 75 % of treated rats showed degenerating neurons in the CA1, CA3 and the hilar region. The degree of damaged neurons was more profound one month after SE in the amantadine treatment group. Treatment of rats with amantadine 60 min post SE did not exert obvious neuroprotective effect at all sections examined.


10 min post SE
60 min post-SE
Control
SE
Per.
Aman.
Per.
Aman.
72 hr. post-SE
1 month post-SE

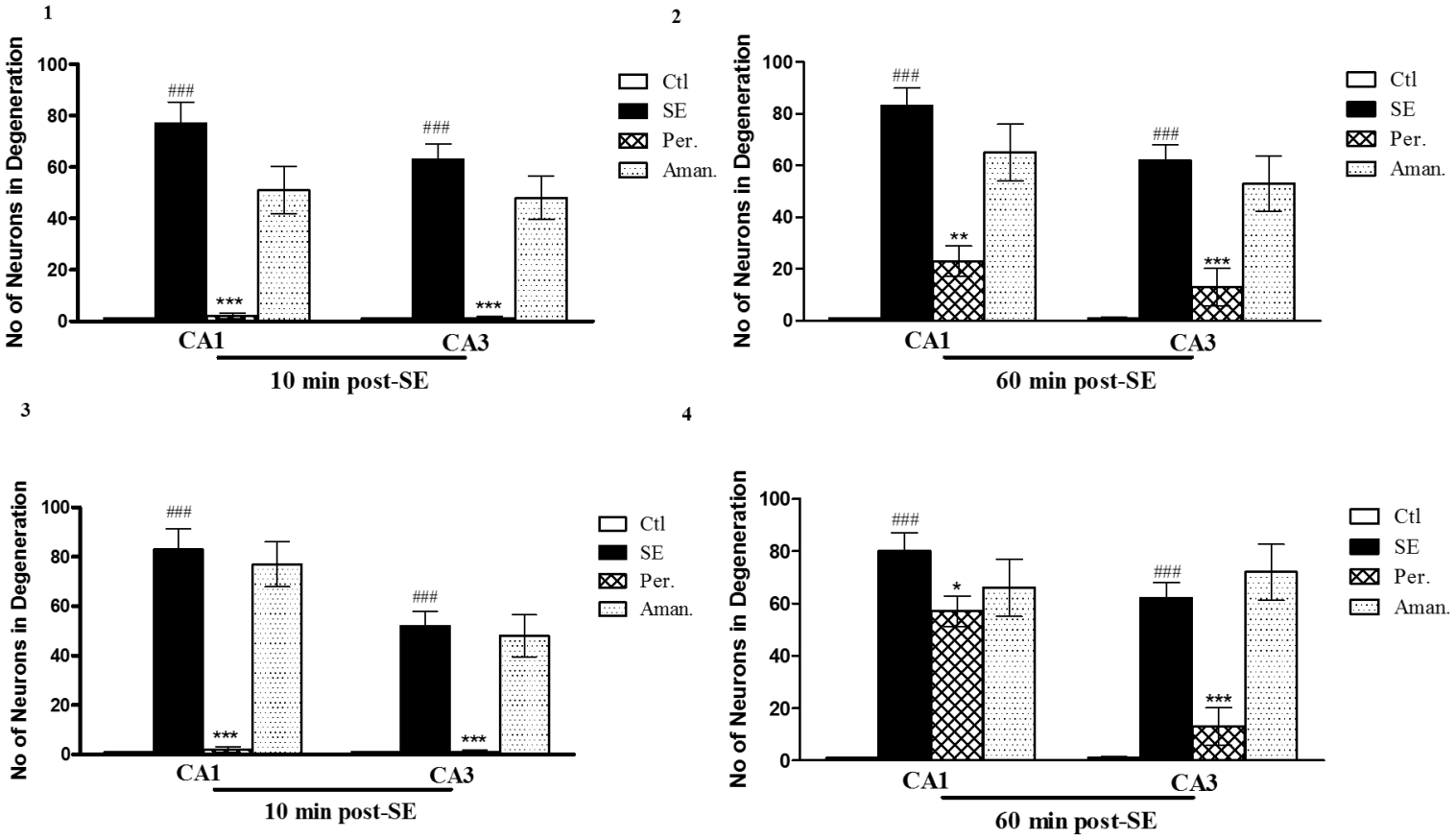
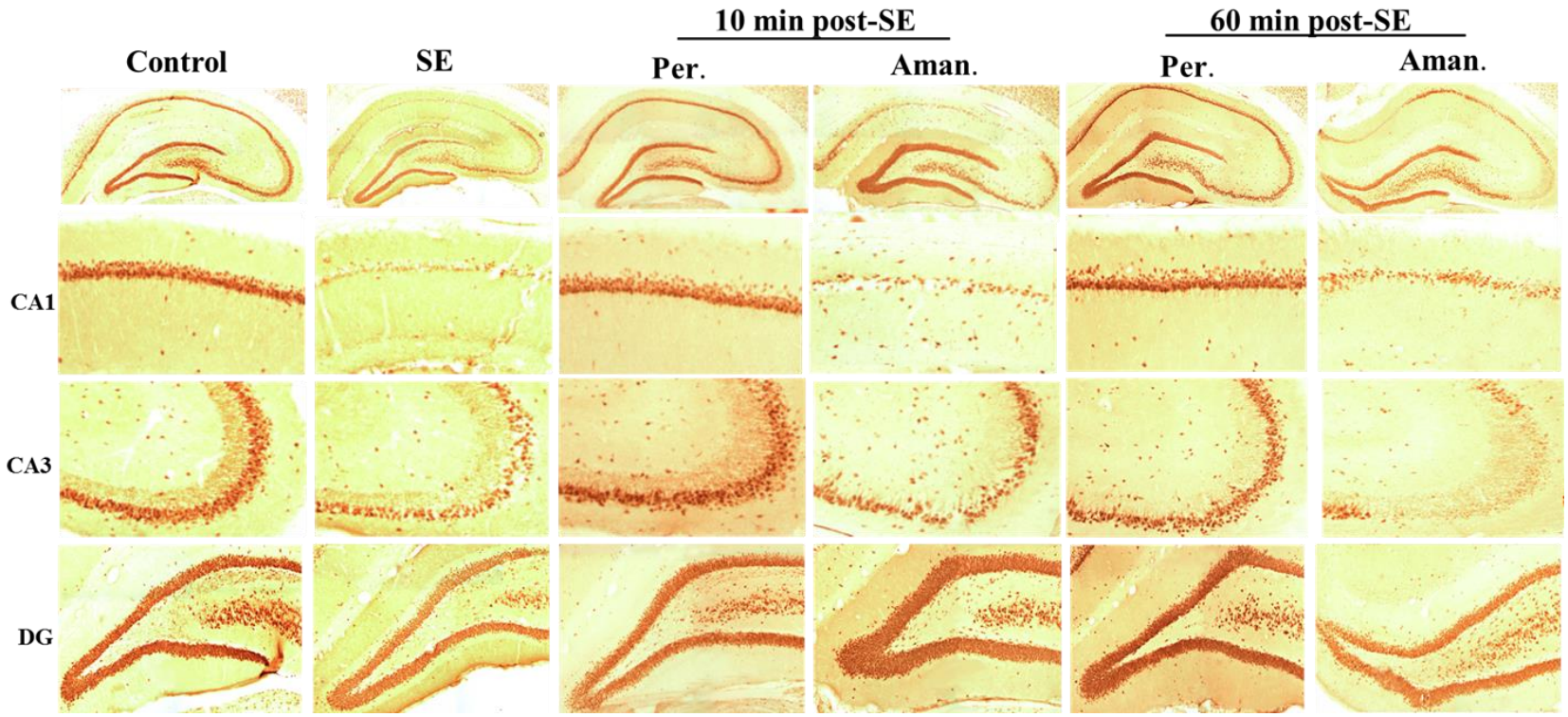
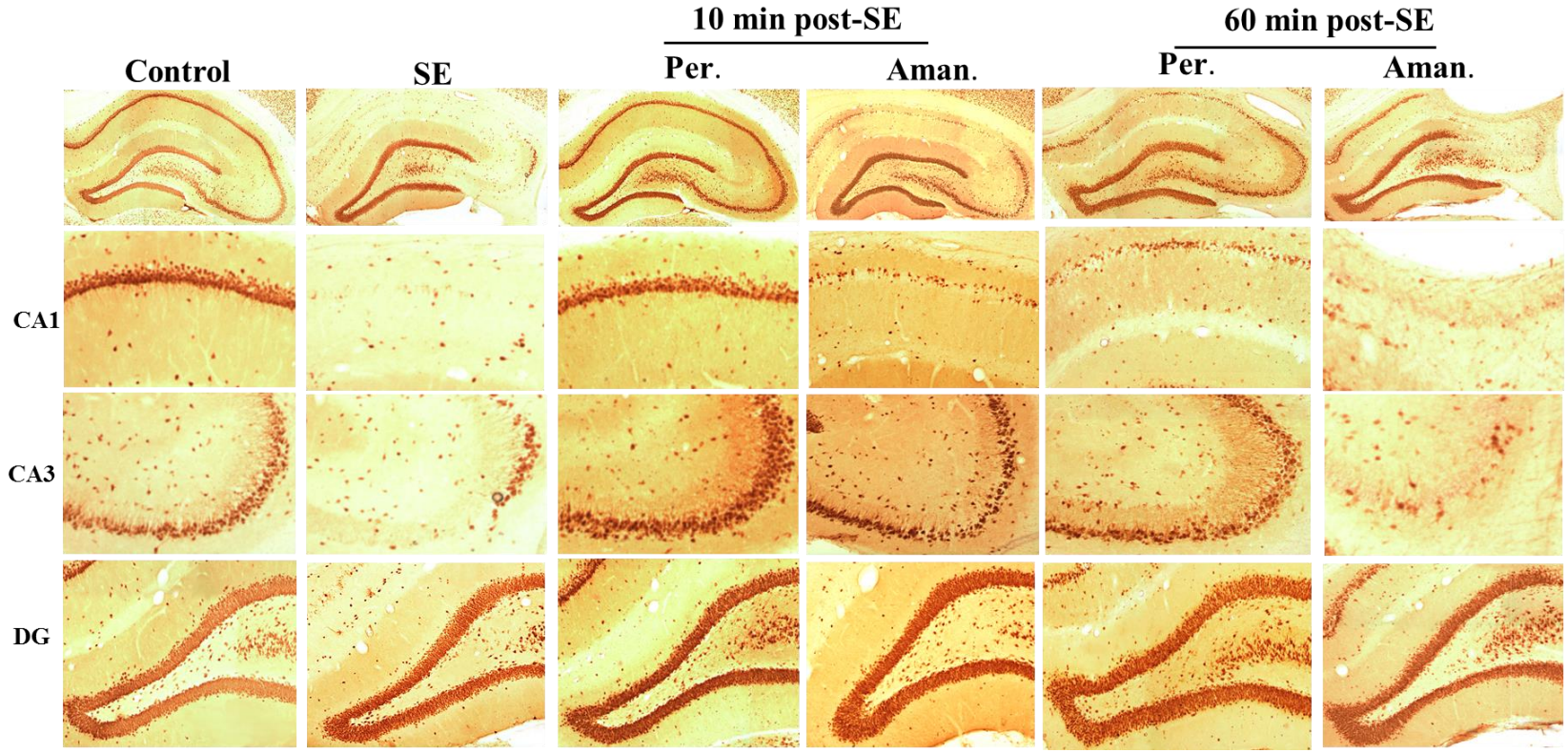

1
No of Neurons in Degeneration
100
80
60
40
20
0
###
###
***
***
CA1
CA3
10 min post-SE
Ctl
SE
Per.
Aman.
2
No of Neurons in Degeneration
100
80
60
40
20
0
###
###
**
***
CA1
CA3
60 min post-SE
Ctl
SE
Per.
Aman.
3
No of Neurons in Degeneration
100
80
60
40
20
0
###
###
***
***
CA1
CA3
10 min post-SE
Ctl
SE
Per.
Aman.
4
No of Neurons in Degeneration
100
80
60
40
20
0
###
###
*
***
CA1
CA3
60 min post-SE
Ctl
SE
Per.
Aman.

Fig. 9. Effects of perampanel or amantadine on neuronal survival in pilocarpine induced SE rat model. Upper panels represented the Fluorojade C (FJC) positive staining in bright green color, indicating degenerated neurons. Representative images of FJC stained hippocampus in control, SE, perampanel (Per.) and amantadine (Aman.)-treated rats at (A) 72 hr. and (B) 1 month after SE induction. Lower panels represented histograms showing number of FJC positive cells in the CA1 and CA3 regions. (1) 72 hours group (treatment started 10 min post-SE); 1-month group (treatment started 10 min post-SE); 72 hours group (treatment started 60 min post-SE); 1-month group (treatment started 60 min post-SE). Values are expressed as mean ± SD. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. ###, $p< 0.001$ vs control. *, **, ***, $p < 0.05$, 0.01, 0.001 respectively vs SE group.


10 min post-SE
60 min post-SE
Control
SE
Per.
Aman.
Per.
Aman.
CA1
CA3
DG


10 min post-SE
60 min post-SE
Control
SE
Per.
Aman.
Per.
Aman.
CA1
CA3
DG

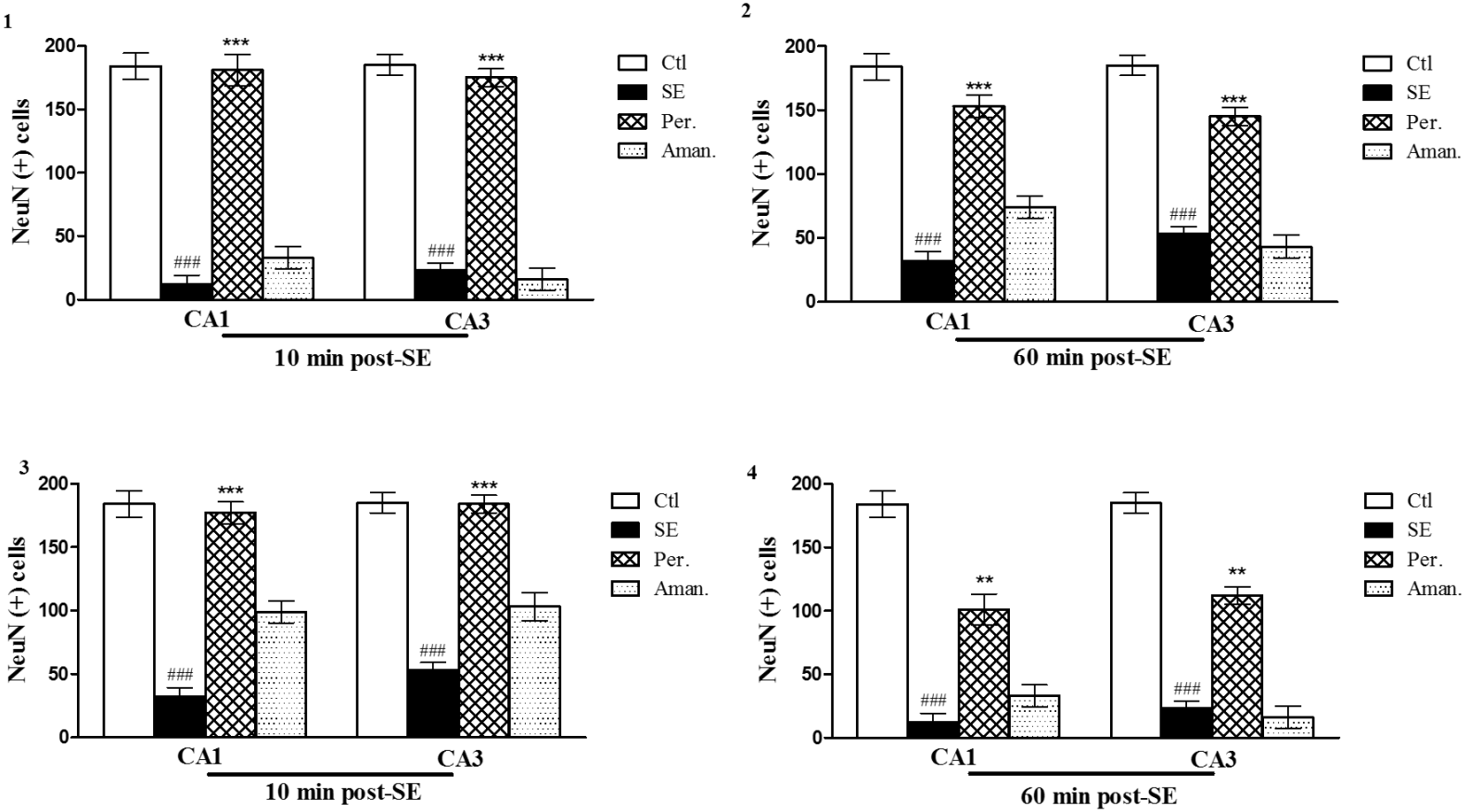
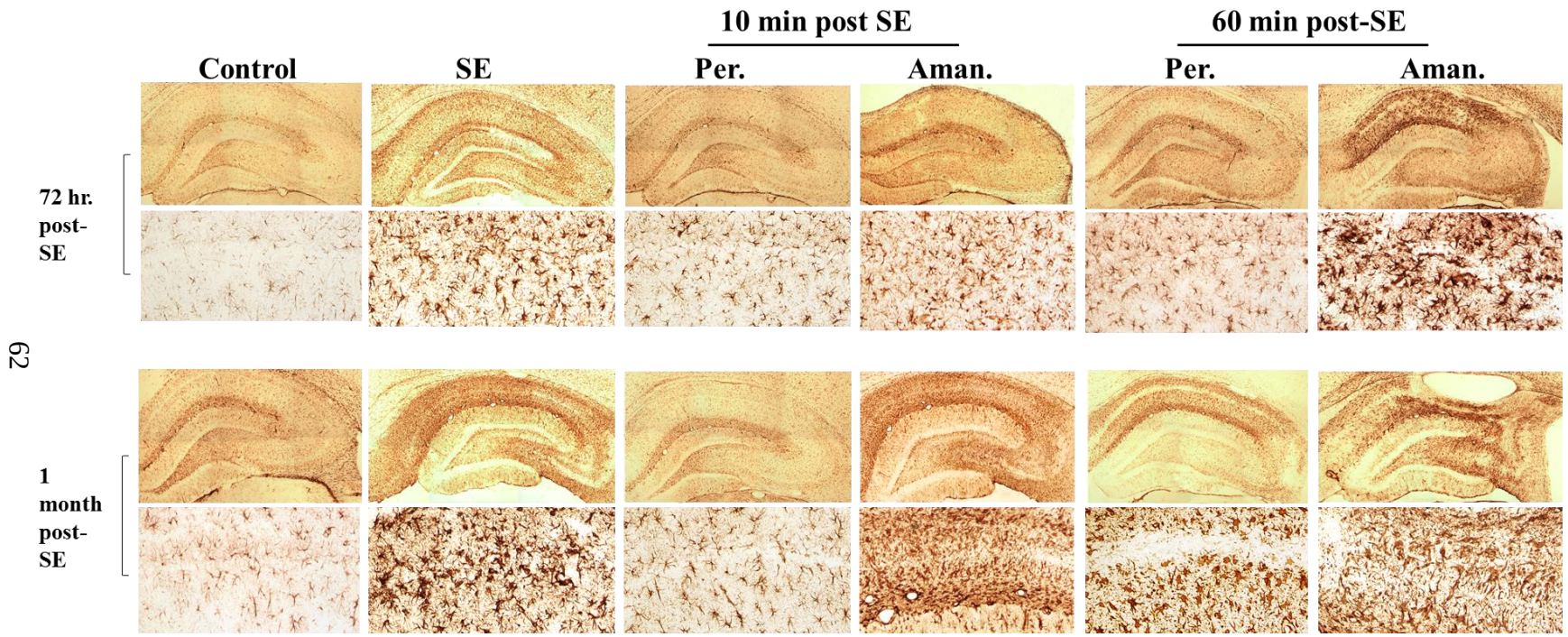

1
NeuN (+) cells
200
150
100
50
0
***
###
CA1
CA3
10 min post-SE
Ctl
SE
Per.
Aman.
2
60 min post-SE
3
10 min post-SE
4
**
60 min post-SE

Fig. 10. Effects of perampanel or amantadine on neuronal nuclei (NeuN) expression in pilocarpine induced SE rat model. Upper panel represented images of NeuN immunohistochemistry of the CA1, CA3 and DG regions of the hippocampus in control, SE, perampanel (Per.) and amantadine (aman.)-treated rats at (a) 72 h and (b) 1 month after SE induction. (C) Lower panel represented histograms showing the number of NeuN immune-positive cells in the CA1 and CA3 regions. (1) 72 hours group (treatment started 10 min post-SE); (2) 1-month group (treatment started 10 min post-SE); (3) 72 hours group (treatment started 60 min post-SE); (4) 1-month group (treatment started 60 min post-SE) Values are expressed mean ± SD. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. ###, $P < 0.001$ vs control group; **, ***, $p < 0.01$ and 0.001, respectively vs seizure group.

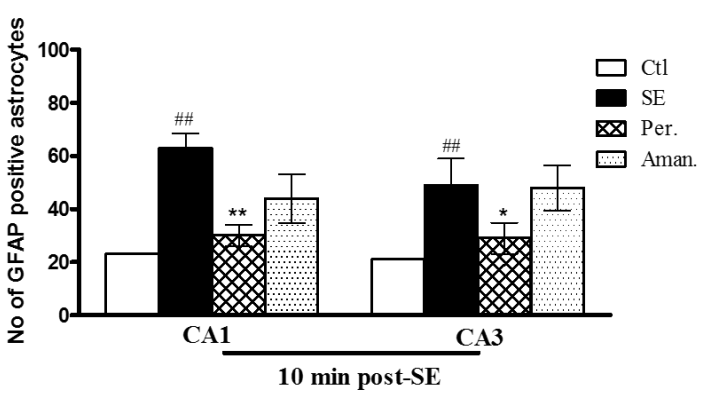
4.2.5. Effect of perampanel and amantadine treatment on the activation of astrocytes.

GFAP is regarded as a marker of reactive gliosis. It is well known that following brain lesions, astrocytes become reactive and release numerous proinflammatory cytokines that play an important part in secondary injury. Control rats showed few GFAP positive cells in the CA1, CA3 and the dentate hilar regions (Fig.11) and these cells had a typical morphology of resting astrocytes. At 72 hours after the induction of SE, the pilocarpine treated seizure group showed profound gliosis demonstrated by higher number of GFAP immunoreactivity. GFAP positive astrocytes showed enlarged soma size (hypertrophy) and longer projections together with increased GFAP expression. Compared to the SE group, the SE + perampanel group (10 min group) had significantly less GFAP positive astrocytes while amantadine treatment 10 min failed to significantly suppress the SE induced gliosis.

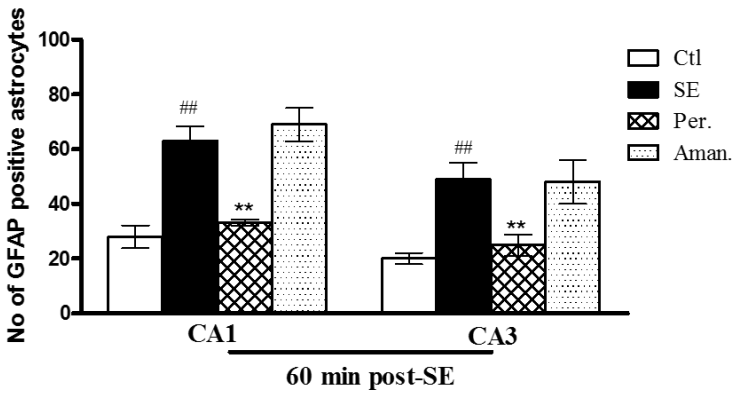
Administration of perampanel 60 min after SE, reduced gliosis from the CA3 and the hilar region, but not from the CA1 area. Compared to the SE group, amantadine treatment 60 min post SE was not effective in reducing the degree of gliosis.


10 min post SE
60 min post-SE
Control
SE
Per.
Aman.
Per.
Aman.
72 hr. post-SE
1 month post-SE

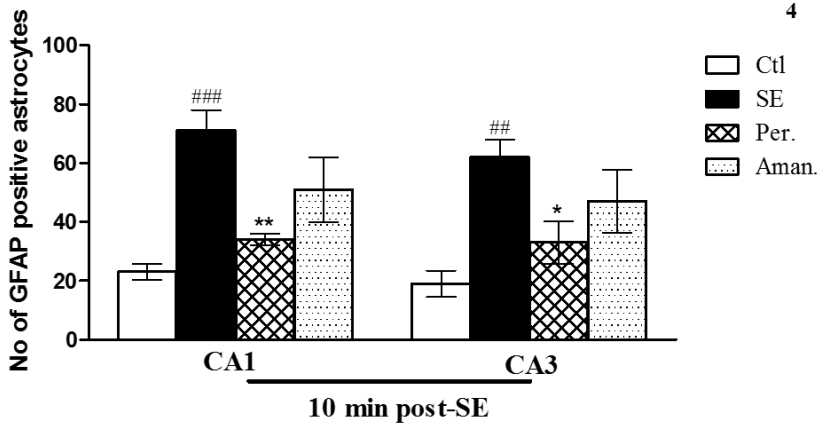
1


No of GFAP positive astrocytes
100
80
60
40
20
0
##
**
##
*
Ctl
SE
Per.
Aman.
CA1
CA3
10 min post-SE

2


No of GFAP positive astrocytes
100
80
60
40
20
0
##
**
##
**
Ctl
SE
Per.
Aman.
CA1
CA3
60 min post-SE

3


No of GFAP positive astrocytes
100
80
60
40
20
0
###
**
##
*
Ctl
SE
Per.
Aman.
CA1
CA3
10 min post-SE

4

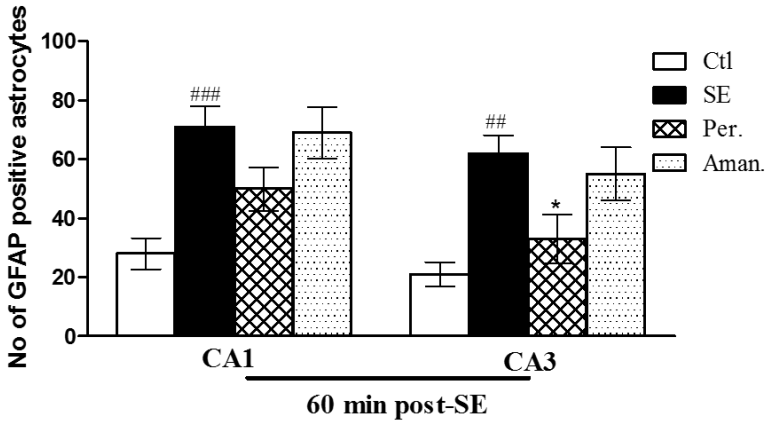
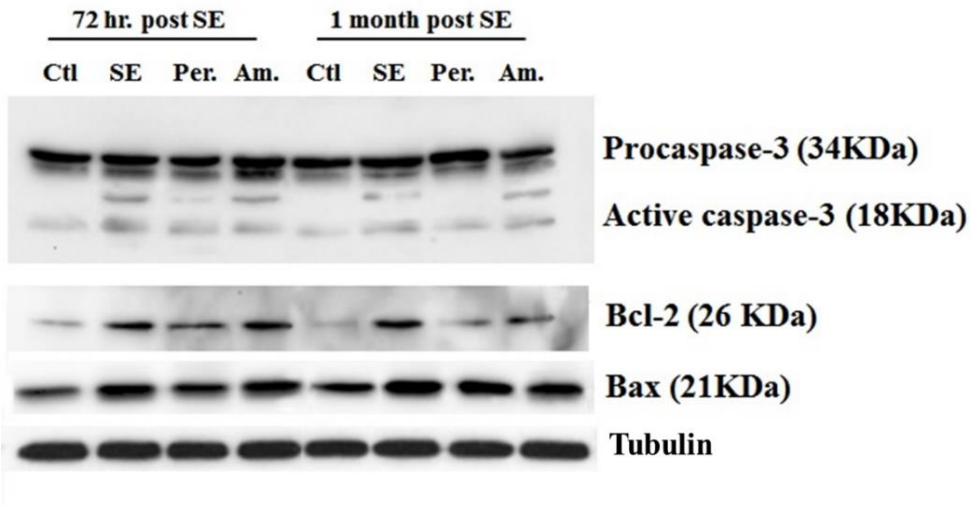

No of GFAP positive astrocytes
100
80
60
40
20
0
###
##
*
Ctl
SE
Per.
Aman.
CA1
CA3
60 min post-SE

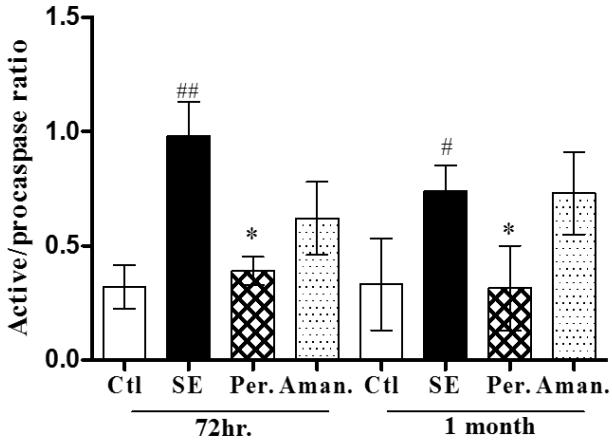
Fig.11. Effect of perampanel or amantadine on astrocyte activation in hippocampus of pilocarpine induced status epilepticus (SE) rat model at 72 h. and 1 month after SE. (A) Representative images of GFAP immunohistochemistry of Lower magnification images (4x) showing the astrogliosis in the entire hippocampus in control, SE, perampanel (Per.) and amantadine (aman.)-treated rats at 72 h and 1 month of SE induction. (B) Graphs showing number of GFAP immune-positive cells in the CA1 and CA3 regions. Values are expressed mean±SD. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison in graph pad prism 5.0. ### represents P value < 0.001 vs control; **, *** represents P value < 0.01, 0.001 respectively vs seizure group.

4.2.6. Perampanel, not amantadine inhibited caspase-3 expression in SE rats.

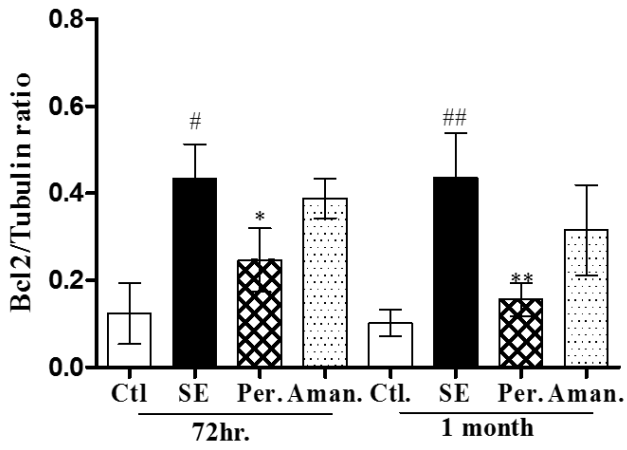
In order to explore the underlined mechanism exerted by perampanel, western blot analysis was applied to detect activated caspase-3 levels in hippocampal region from all treatment groups. The immunoreactivity of caspase-3 cleaved bands at 18 kDa, representing active caspase-3, was increased in hippocampal regions from SE rat brains ($P < 0.001$); this enhanced expression was significantly reduced by perampanel, not amantadine treatment (Fig.12).


72 hr. post SE
1 month post SE
Ctl SE Per. Am. Ctl SE Per. Am.
Procaspase-3 (34KDa)
Active caspase-3 (18KDa)
Bcl-2 (26 KDa)
Bax (21KDa)
Tubulin

1


Active/procaspase ratio
1.5
1.0
0.5
0.0
##
#
*
*
Ctl SE Per. Aman. Ctl SE Per. Aman.
72hr.
1 month

2


Bcl2/Tubulin ratio
0.8
0.6
0.4
0.2
0.0
#
*
##
**
Ctl SE Per. Aman. Ctl. SE Per. Aman.
72hr.
1 month

3

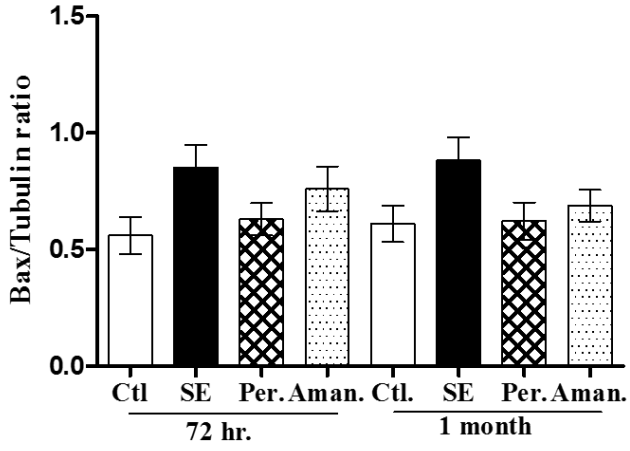

Bax/Tubulin ratio
1.5
1.0
0.5
0.0
Ctl SE Per. Aman. Ctl. SE Per. Aman.
72 hr.
1 month

Fig. 12. Effect of perampanel or amantadine on apoptotic markers in pilocarpine induced status epilepticus (SE) rat model. Representative western blot showing the expression levels of caspase 3, bcl2 and bax protein in control, vehicle SE, perampanel and amantadine treated rats at 72h and 1 month after the induction of seizure. Graphs showed the changes of those protein expression in different treatment groups; (1) Active/proactive caspase-3 ratio; (2) Bax/tubulin ratio; (3) Bcl2/tubulin ratio. Data are expressed mean ± SD. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. #, ###, $p < 0.05$ and 0.001, respectively vs control group. *, $p < 0.05$ vs SE group.

4.2.7. Side effects of perampanel and amantadine treatment after SE

i. Neurological responsiveness:

In clinical settings, the reliable assessment of a patient's neurological status becomes problematic under the influence of benzodiazepines or barbiturates due to their sedative and muscle relaxant effects. AMPA antagonists are also known to have mild sedative and muscle relaxant side effects. In order to determine whether perampanel and amantadine administered under the treatment conditions used in these experiments produced impairment of neurological responsiveness, we assessed the responsiveness of the mice to mechanical stimulation of the vibrissae 10 min after the treatment bolus. Perampanel-treated rats exhibited clear sedation and immobility. Nevertheless, 80% of rats treated with perampanel showed a motor reaction upon mechanical stimulation of the vibrissae. Rats in the amantadine treated group and the SE group continued to exhibit motor seizures and were not tested for responsiveness to mechanical stimulation as seizure-related and reflex movements were not distinguishable.

ii. Body temperature:

The adverse effects observed after perampanel and amantadine following SE were more severe than observed in the perampanel and amantadine control rats without SE. All SE rats showed slight increase in body temperature (38 ± 1.4 °C; values expressed in mean±SD). Perampanel treated animals after SE showed decrease in body temperature (34.2 ±1.3 °C; values expressed in mean±SD) and sedation. Two perampanel treated rats died within 2 hours following induction of SE. However, none of perampanel treated rats in the control group showed no significant decrease

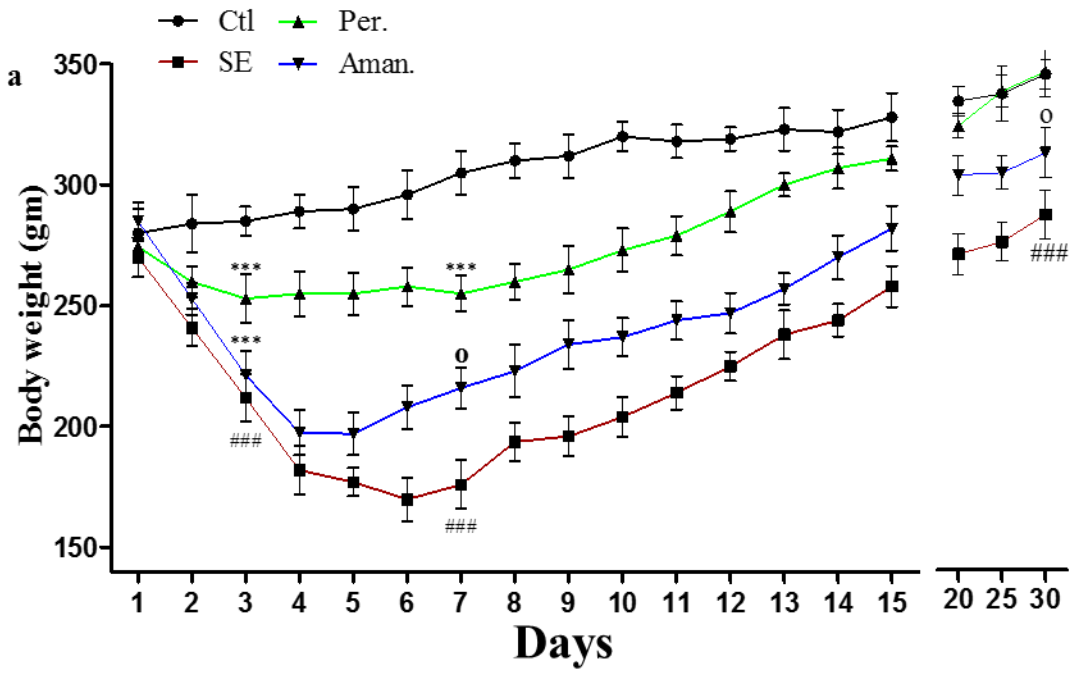
in body temperature. Amantadine treated rat showed a slight elevation of body temperature similar to the SE group.

iii. Body weight:

With respect to the changes of body weight, it has to be noted that, after the induction of SE, the general condition of all rats was severely impaired, including a decrease in body weight (Fig.13a). All the rats after perampanel treatment lost some weight, but it was not significant compared to the non-epileptic control group. Interestingly, amantadine seemed to counteract, at least in part, the loss of body weight after SE (Fig.13a.). During the 1 month after SE, the body weight of rats treated with amantadine was significantly higher compared to SE vehicle rats at 1-week post-SE. However, this significance was lost during the following weeks. When body weight of the SE and the amantadine group was compared to the non-epileptic control rats, both were significantly different from the control by the end of the treatment.

iv. Mortality:

As illustrated in (Fig.13b) , vehicle treated animals that had received a 380 mg/kg dose of pilocarpine exhibited 17% mortality. The mortality rate in animals receiving perampanel at 10 min was greater (8.3 % mortality) than in the vehicle group whereas the animals receiving diazepam at 60 min did not show mortality. In a pilot experiment (n=4), all rats treated with amantadine only after 10 min of SE onset died within 12 hours. The administration of pentobarbital increased the survival rate and all the amantadine treated rats after SE survived (Fig.13b)


a
Ctl
Per.
SE
Aman.
Body weight (gm)
350
300
250
200
150
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 20 25 30
Days
***
***
***
o
o
###
###
###

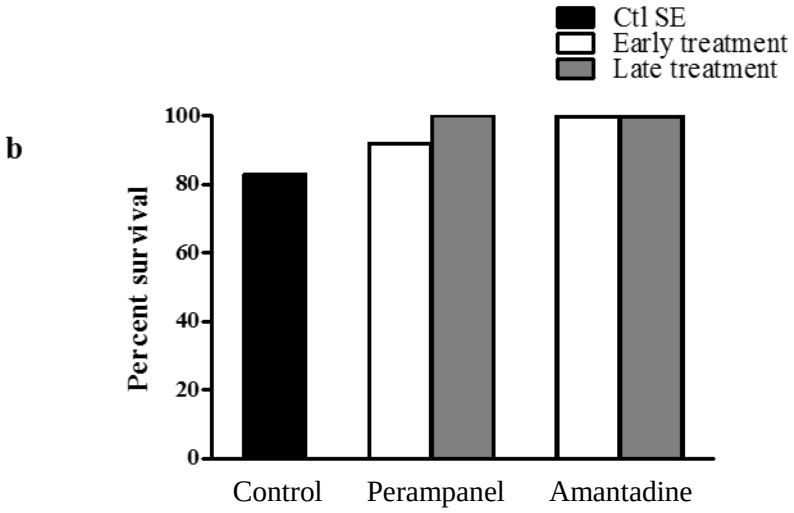

b
Ctl SE
Early treatment
Late treatment
Percent survival
100
80
60
40
20
0
Control
Perampanel
Amantadine

Fig. 13. Effect of perampanel or amantadine on body weight and survival rate in pilocarpine induced status epilepticus (SE) rat model. (a) Body weight of rats in the control (n=24), SE (n=24), perampanel (n=24) and amantadine (n=23) groups after the induction of SE. Data are means ± SEM. the body weight determined in sham controls on day 3 and 7 and 30 post-SE was used for statistical comparison. Mean difference between the groups were analyzed using one-way ANOVA followed by tukey's multiple comparison test in graphpad prism 5.0. ### Significant differences ($P < 0.001$) between the SE group and the control. *** indicated significant differences between the perampanel group against the SE group ($P < 0.001$). The difference between the amantadine and the SE was indicated by circle ($P < 0.05$). (b) Comparison of percent survival in the different treatment groups.

5. Discussion:

The present study investigated the hypothesis that perampanel and amantadine possessed therapeutic effects on status epilepticus (SE). Our data showed that perampanel could terminate ongoing status epilepticus providing a long-lasting inhibition of the seizure whereas amantadine failed to terminate the seizure after the development of SE. Furthermore, for the long-term consequences of SE, i.e., the cognitive impairment, development of SRS and hippocampal damage in the pilocarpine rat SE model, perampanel prevented memory decline, retarded the appearance of SRS and reduced SE induced hippocampal cell death. In contrast to our expectations, the consequences of the treatment with amantadine after SE were largely negative. SE rats treated with saline or amantadine developed SRSs. Memory impairments were observed in both groups with significant cell loss in the hippocampal formations compared to controls.

It has been shown that prolonged seizures can lead to neurological and systemic complications, including pulmonary congestion and edema, cardiac arrhythmias, hypotension, elevation of body temperature, hypoglycemia, acidosis and rhabdomyolysis (186) (187). These systemic complications tend to be exacerbated as the duration of SE prolonged (188) (189). Refractoriness of SE to benzodiazepines also worsens with the passage of time (190). Mortality associated with status epilepticus also correlates with the duration of the epileptic seizures (191). We first chose to administer the experimental drugs 10 min post-SE as it is important to terminate SE in a prompt manner. As shown in the present study, early administration of perampanel successfully terminated ongoing seizure. These results were in agreement with previous studies of AMPA receptor antagonists in animal models of SE (192) (193) (194) (195). Our data further showed that early administration of perampanel successfully prevented the memory deficit and the neuronal loss 72

h and even 1 month after SE. Neither of the rats after early treatment with perampanel developed epilepsy in the chronic period. Our data indicated that perampanel exerts the neuroprotective effects by interfering with the initial insult and that the long-term consequences of SE can be diminished by reducing the severity or the duration of the seizure (192).

We next investigated whether late treatment with competitive AMPA receptor antagonist will prevent the long-term impairment caused by SE. In the pilocarpine rat model, a duration of 60-90 min SE is needed to induce epileptogenesis and brain damage in the majority of rats (155). There was an increase in the latency to terminate the seizure when perampanel was given 60 min post-SE suggesting a tolerance against perampanel treatment developed. In addition, we found that the late administration of perampanel is not as efficacious in protecting CA1 neurons against seizures insults as early intervention. It is potentially due to a more sensitive response to AMPA receptors or AMPA receptors may not be the primary mediator of seizure to induce cell loss, at least within the CA1 neurons as the seizure progresses. When perampanel was given 60 min after the induction of SE, 2 out of 10 rats developed SRS. The frequency of seizure was less than that in the SE control rats. It is possible that treatment with perampanel has prevented or delayed the development of epilepsy, or that the rats with SRS experienced more severe SE than the others. Our data showed that late administration of perampanel exerted antiepileptic efficacy, but not as efficient as early intervention, indicating that other pathways exist in the progression of SE.

Amantadine is an antiviral and antiparkinsonian drug. It also an uncompetitive NMDAR open channel blocker (167). Studies have shown that amantadine has potential neuroprotective properties with a low incidence of side-effects. Wang (2013), found that amantadine improved the cognitive function and reduced the neuronal loss after traumatic brain injury in rats. Several studies have demonstrated that NMDA antagonists are effective as a therapeutic intervention for the

treatment of status epilepticus (196) (197) (198). However, amantadine was the least effective drug tested in this study. At a dose of 45 mg/kg, neither 10 nor 60 min after SE onset, amantadine showed its ability to terminate SE. In contrast, amantadine increased the intensity of behavioral and electrographic seizure. To our knowledge, the mechanism by which amantadine exacerbated the seizure is unknown. This lack of anticonvulsant efficacy of amantadine in the pilocarpine rat model was in agreement with some studies that have demonstrated that NMDA antagonist may lack potency during the early stage of SE (199) (127).

Most of the amantadine treated SE rats developed SRS and showed no significant improvement in exploring the novel arm in the Y maze test compared to the SE rats. Histology analysis showed that amantadine slightly decreased the neuronal loss in the 72-h but not in the 1-month duration. The lack of efficacy of amantadine treatment might be due to the ability of glutamate released during the seizure to replace amantadine from its binding sites of NMDA receptors. Another explanation for the lack of antiepileptogenic or a significant neuroprotective effect of amantadine might be that the doses administered were low. Amantadine is more rapidly eliminated in rats than in humans (1.2 vs. 18 h, respectively) (200). In our pilot study conducted to adjust the dose of amantadine (data not shown), higher doses or more frequent administration of amantadine increased the aggressive behavior in treated rats which limited this application. Nevertheless, amantadine showed some additive effect in terminating the seizure when combined with perampanel (data not shown).

The impact of pilocarpine induced seizure on the brain is controversial (201) (202). Researches have demonstrated that seizure damage neuronal cells by a necrotic or programmed cell death pathway (203) (204). In addition, some studies have identified upregulation of bcl-2 family gene, DNA fragmentation and some morphological features of programmed cell death (205) (206) . Bcl-

2 is known to be an antiapoptotic molecules (207). In the present study, we identified significantly higher levels of bcl-2 in epileptic brains compared to the control. These data suggest that neuronal cells respond to seizure stress by upregulation of this cell death suppressor gene. This was confirmed by previous experimental reports that detected elevated bcl-2 levels in neurons in human temporal lope epilepsy samples and experimentally induced ischemia, trauma and seizure (208) (209) (210) (211). Caspase-3 cleavage was also observed in the SE brains. This finding supports studies of caspase-3 activation following experimentally induced SE in different animal models (212) (213) (214) (215). Our data indicate that programmed cell death is activated in pilocarpine induced status epilepticus and may contribute to SE neuronal death. Perampanel, reducing the seizure intensity, most likely inhibited this apoptotic pathway.

Using an in vitro primarily cultured rat hippocampal neuron, our present study demonstrated that perampanel and amantadine could partially reverse cytotoxicity and inhibit the activation of caspase-3 induced by AMPA and NMDA, respectively. Western blot showed that perampanel and amantadine could partially reduce levels of GAPDH, p53, PTEN, and active SREBP-1 expressed in nuclear fractions of cultured hippocampal neurons treated with AMPA and NMDA, respectively. There is a considerable body of evidence that nuclear translocation of GAPDH is involved in the pathogenesis of neuronal cell death (216) (217). GAPDH is also involved in the apoptotic process (218). Wang (2012), have suggested that GluA2 forms a protein complex with GAPDH and it is co-internalized upon activation of AMPA receptors. The observed increases in the levels of nuclear GAPDH hippocampus after AMPA or NMDA excitotoxicity may be due to GluR2/GAPDH complex formation that would promote increased hippocampal apoptosis. Also, we found higher expression of p53 in nuclear fractions after induced excitotoxicity that may trigger

apoptotic events (219). Furthermore, nuclear translocation of GAPDH forms a protein complex with the activated form of p53 and initiates cell death as previously reported (220).

The full-length form of lipid transcription factor, SREBP1 (125KDa) is predominantly expressed in the endoplasmic reticulum. However, in conditions of cellular stress and hypoxia, the full-length SREBP1 is cleaved and truncated to an activated form of SREBP1 [65KDa] which enters the nucleus to induce neuronal excitotoxicity. The data from the present study shows that the cleaved SREBP1 (65KDa) but not the full-length 125KDa SREBP1 is elevated in NF AMPA and NMDA treated cultures, which confirms it may contribute to the initiation of hippocampal cell death. Previous studies using animal models of hypoxic stroke and amyotrophic lateral sclerosis and human brain tissues have shown that neuronal excitotoxicity is linked to increased level of SREBP1 (65KDa) and disruption of this mechanism reduces brain injury (221) (222). The results from the present study support the theory that neuronal cell accumulation of SREBP1 (65KDa) could contribute to cell death. While the increase in SREBP1 level failed to reach statistical significance in the NF of AMPA/NMDA treated cells, the possibility that SREBP1 may contribute to cell death cannot be ruled out, and more studies are warranted to address its participation in excitotoxicity.

6. Conclusion:

In conclusion, our data demonstrates that perampanel is an effective drug to suppress focal electrographic seizures in a pilocarpine rat model of Status Epilepticus. In addition, Prophylactic administration of perampanel after SE suppressed the SE-induced neuronal loss and the development of Spontaneous Recurrent Seizures (SRSs). The neuro-protective efficacy of perampanel makes this antiepileptic drug an interesting tool to examine its value in the long-term management of SE-induced neuronal damage in human patients. While it is not possible to draw a clinical conclusion from this animal study, our results support the design of future clinical studies to assess the role of early administration of perampanel in human SE to save hippocampal-dependent memory function. In contrast to our expectation, treatment with amantadine was largely negative without significant effect on the development of SRSs, behavioral alteration or hippocampal damage. This does not mean that amantadine is not a potentially interesting drug in the treatment of SE. For instance, amantadine was found to be neuroprotective in a TBI animal model. It also showed some additive effect in terminating the seizure when combined with pentobarbital. To our knowledge, this is the first study to investigate the long term neuroprotective efficacy of perampanel and amantadine in SE-rat model. The present study opens a new vista in the consideration of these drugs from the “bench to the bed side” as they likely hold promise in offering neuro-protection in brain insults such as SE, stroke and head trauma.

7. Future perspectives:

1. To determine whether a combination of amantadine and perampanel can be a potential treatment of Status Epilepticus.
2. To use synchrotron technology to characterize the neuroprotective effect(s) of glutamate receptor antagonists on the hippocampal degeneration typically induced by SE.
3. To investigate the insulin signaling alterations in SE.
4. To use PET scanning in order to investigate glucose metabolism in Status Epilepticus.

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