Alteration of astrocyte markers and phosphorylation of tau protein in kainic acid-treated animal model of Temporal Lobe Epilepsy

by

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ABSTRACT

Kainic acid, an analogue of the excitatory neurotransmitter glutamate, when administered systemically can trigger seizures and neuronal loss in a manner that mirrors the neuropathology of human mesial temporal lobe epilepsy (mTLE), which affects ~50million people globally. Evidence suggests that changes in astrocytes which precede neuronal damage play an important role in the degeneration of neurons and/or development of seizures in TLE pathogenesis. Additionally, a role for microtubule associated tau protein, involved in various neurodegenerative diseases including Alzheimer's disease, has also been suggested in the development of seizure and/or neurodegeneration in TLE pathogenesis. At present, possible alterations of different subtypes of astrocytes and their association, if any, with tau protein in TLE remain unclear. In this study, we evaluated alterations of different subtypes of astrocytes and phospho-/cleaved-tau levels in kainic acid-treated animal model of mTLE. Our results reveal that levels/expression of various astrocytes markers such as GFAP, vimentin, S100β, Aldh1l1, but not GS, are differentially altered in the hippocampus of kainic acid-treated rats. The levels/expression of both A1(C3+) and A2(S100A10+)-like astrocytes are variably increased in kainic acid-treated rats. The hippocampal levels of total (Tau1 and Tau5) and phospho-tau (AT270 and PHF1) proteins are also transiently enhanced following kainic acid administration. Furthermore, the steady-state level and expression of cleaved-tau, which is evident in a subset of GFAP-positive astrocytes, are found to be increased in kainic acid-treated rats. These results, taken together, suggest a differential role for various astrocytic subpopulations and tau protein in kainic acid model of TLE and possibly in human mTLE pathogenesis.

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Abhishek Dahal

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ABBREVIATIONS

Aβ	Amyloid-β peptide
AD	Alzheimer's disease
Aldh1l1	Aldehyde Dehydrogenase 1 Family Member L1
AMPA	alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BSA	Bovine serum albumen
C3	Complement Protein C3
DAB	3,3'-Diaminobenzidine
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethelynediaminetetracetic acid
ELISA	Enzyme-linked immunosorbence assay
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GS	Glutamine Synthetase
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
KA	Kainic acid
MAPT	Microtubule-associated protein tau

MTLE	Mesial Temporal lobe epilepsy
NFT	Neurofibrillary tangles
NMDA	N-methyl D-aspartate
PBS	Phosphate-buffered saline
PHF	Paired helical filament
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecippitation assay buffer
SDS	Sodium dodecyl sulfate
S100β	S100 Calcium binding protein B
S100A10	S100 Calcium Binding Protein A10
TBI	Traumatic Brain Injury

1. INTRODUCTION

1.1 *Epilepsy*

Epilepsy, the third most diagnosed neurologic disorder, is characterized by the occurrence of spontaneous recurrent seizures resulting from abnormal, excessive discharge of neurons located in defined regions of the brain. It affects ~50 million people worldwide and its prevalence increases with age, especially in population with dementia (Avoli et al., 2005; Majores et al., 2007). For a century, fear, confusion, discrimination, and social stigma have surrounded this condition, affecting the quality of life of those who are afflicted and those who care for them (World Health Organization, 2019).

The state of having repeating episodes of abnormal neuronal activity known as seizures, in which groups of neurons within specified brain areas fire unusually in coordinated, repetitive bursts, is believed to be the underlying cause of epilepsy. Seizures can be triggered by a variety of factors, such as a reaction to an insult or damage that causes a reconfiguration of the neuronal circuit to favour hyperexcitability (Hirtz et al., 2007; Scharfman, 2007). Seizures are classified as partial or general, depending on whether they are isolated to a certain brain area or spread throughout the brain regions and affect the entire brain. Generalized seizures cause a loss of consciousness and motor control, which can range from involuntary twitching (myoclonus) to convulsions (tonic-clonic seizures). On the other hand, partial seizures might progress to generalised seizures. Neuronal loss, gliosis and synaptic plasticity are all possible outcomes in this brain location. At present, pharmacotherapy (i.e., antiepileptic drugs such as carbamazepine, topiramate, gabapentin, lamotrigine, oxcarbazepine etc.) represents the mainstay of treatment for epilepsy patients, whereas resective surgery and vagal nerve stimulation are other options for selected patients whose

seizures cannot be controlled by existing drugs. Notwithstanding these therapeutic strategies, a significant number of epilepsy patients continue to live with uncontrolled seizures, often also involving significant drug-induced adverse effects. Over time, this leads to a decrease in cognitive functions and a poorer quality of life. These facts, together with a steady increase in life-expectancy as well as the prevalence of dementia throughout the world, make epilepsy one of the serious health problems of our time (Sundqvist, 2002; Wieser, 2004; Cloyd et al., 2006; Kohrman, 2007; Hommet et al., 2008).

1.2 *Temporal Lobe Epilepsy (TLE)*

Of the various forms of epilepsies, Temporal Lobe Epilepsy (TLE) is the most common which originates from the hippocampus and then propagates to other limbic areas such as the amygdala and entorhinal cortex. The most predominant feature associated with human TLE patients is hippocampal sclerosis - a distinct pathological entity with a typical histopathological and clinical history. The clinical account often includes an initial injury followed by a latent period of structural and functional changes in the hippocampus that lead to a reduction of the seizure threshold and the development of chronic spontaneous seizures (Blümcke et al., 2002; Thom, 2004; Dawodu and Thom, 2005). At the pathological level, hippocampal sclerosis is characterized by atrophy, induration and loss of neurons in CA1, CA3 and the dentate hilar regions, whereas CA2 and dentate gyrus granule cells are relatively spared. This is accompanied by gliosis and reorganization of mossy fiber synapses, which facilitate the generation of epileptiform activity in the hippocampus. In many TLE patients the entorhinal cortex and amygdala are also affected by cellular damage due to propagation of seizures from the hippocampus (Blümcke et al., 2002; Thom, 2004; Dawodu and Thom, 2005; Hirtz et al., 2007). Studies from animal models as well as TLE patients have indicated that an excess extracellular levels of glutamate in the sclerotic hippocampus play a critical role in

initiating seizures and loss of neurons in TLE. The glutamate which is the primary excitatory neurotransmitter in the brain is known to mediate its effects by interacting with three ionotropic [i.e., N-methyl D-aspartate (NMDA), alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropionic acid (AMPA) and kainate] and eight metabotropic (i.e., mGluR1-mGluR8) receptors (Kew and Kemp, 2005). A role for glutamate in TLE is supported by the evidence that i) extracellular glutamate levels are increased in the epileptogenic *vs* the non-epileptogenic human hippocampus (Cavus et al., 2005), ii) extracellular hippocampal glutamate markedly increases over the interictal level in humans during and after the seizure (During and Spencer, 1993; Eid et al., 2008), iii) glutamate or its analogues, following administration into animals, can induce seizures and loss of neurons similar to human TLE (Mattson, 2000) and iv) glutamate receptor antagonists such as MK801 and ketamine are effective in reducing seizure-induced neuronal loss in animal models of TLE (Kelsey et al., 2000; Mattson, 2000; Eid et al., 2004).

1.3 *Kainic acid model of TLE*

To better understand the disease pathology, it is important to use experimental models that can recapitulate TLE features. In fact, various experimental models such as kindling, pilocarpine (a cholinergic agonist) or kainic acid-treated animal models are used to study TLE pathology. The major drawback of kindling model is that it does not trigger hippocampal sclerosis, or the lesions associated TLE, raising concerns about the reliability of the kindling model to TLE. This model is also laborious due to repetitive electric stimulation of the animal. Pilocarpine model, on the other hand, exhibits high mortality rate and drastic loss of neurons during latent phase (Lévesque and Avoli, 2013). Thus, kainic acid-induced seizure model is the most commonly studied experimental model for TLE as it can reliably produce acute seizures followed by loss of neurons in CA1/CA3 regions, astrogliosis and reorganization of mossy fibers that closely resemble the “hippocampal

sclerosis" of human TLE (Lévesque and Avoli, 2013). Kainic acid is a non-degradable glutamate analogue and a powerful excitotoxin discovered from in the algae Digenea Simplex. The word "Kainic" comes from the Japanese word "Kaininso" (Makuri), which literally means "sea ghost", isolated first from seaweed *Digenea simplex* (Mody, 1998). Kainic acid was labelled "excitotoxic" by Olney, 1979 and Nadler, 1979 when they revealed that it selectively destroys distinct neurons (Nadler, 1979; Olney et al., 1979). Following the discovery of kainic acid's epileptogenic qualities by Ben-Ari and his colleagues, a plethora of studies have been conducted with an aim to understand TLE pathogenesis. After receiving kainic acid, the animals display wet dog shakes, facial motor signals and chewing, paw tremor related with rearing and falling, and other motor manifestations over a variable time. The seizures usually start in the CA3 region of the hippocampus and then spread to other areas according to electroencephalogram (EEG) recordings. This is followed by astrogliosis and loss of neurons as observed in TLE patients (Ben-Ari et al., 1980; Kar et al., 1997; Ben-Ari and Cossart, 2000; Coulter et al., 2002; Wang et al., 2005; Dudek et al., 2006; Vincent and Mulle, 2009; Giblin and Blumenfeld, 2010; Ben-Ari, 2012; Lévesque and Avoli, 2013; Falcón-Moya et al., 2018). Both the intracerebral and systemic administration of kainic acid provide comparable results; the key difference being the neuropathological damage generated by intracerebral injection is much more rapid and severe than systemic administration. Thus, systemic route of systemic administration is typically used to investigate the selective vulnerability of neurons and the occurrence of more generalized epileptic illness (Lévesque and Avoli, 2013; Grone and Baraban, 2015).

1.4 *Mechanism of action of kainic acid*

Kainic acid, following administration, activates kainate receptors, which are predominantly found in mossy fibres composed of different combinations of five different subunits: Glur5, Glur6, Glur7,

KA1 and KA2 (Ben-Ari, 2012). The activation of kainate receptor leads to the influx of Ca^{2+}, production of reactive oxygen species (ROS) and mitochondrial dysfunction leading to neuronal death. Studies from animal models and TLE patients have suggested that recurrent seizures resulting from neuronal hyperactivity, mediated partly *via* excitotoxic glutamatergic transmission, play a critical role in triggering death of neurons in TLE (Mattson, 2003; Cavus et al., 2005; Eid et al., 2008). More recently, some studies have shown that reactive astrocytes, by regulating uptake/release of glutamate, can also contribute to the generation of seizures and loss of neurons in the hippocampus (Eid et al., 2008; Vincent and Mulle, 2009; Seifert et al., 2010; Foresti et al., 2011). However, very little is known about the functional interrelationships between neurons and glia and their implications in the development of TLE.

1.5 *TLE and Tau protein*

There is evidence that the incidence of unprovoked seizure is significantly higher in patients with Alzheimer's disease (AD), the most common etiology for dementia, than in age-matched reference populations. The relationship appears to be stronger in autosomal dominant early-onset AD cases which are caused by mutations in either amyloid precursor protein (APP), presenilin 1 (PS1) or PS2 genes than in sporadic cases (Selkoe and Hardy, 2016; Chen and Mobley, 2019). Notwithstanding the etiology, the neuropathological features associated with AD include the presence of intracellular neurofibrillary tangles (NFT) containing hyperphosphorylated microtubule associated tau protein, extracellular neuritic plaques containing β-amyloid (Aβ) peptide and the loss of neurons in defined regions of the brain (Ballard et al., 2011). Interestingly, some epilepsy patients are found to exhibit Aβ-containing plaques in the temporal region of the brain as observed in AD pathology (Mackenzie and Miller, 1994; Sima et al., 2014; Gourmaud et al., 2020), thus suggesting a potential role for the peptide in the development of seizure and/or

disease pathology. This is partly supported by results which showed that: i) Aβ can enhance susceptibility of neurons to kainic acid-induced toxicity (Morimoto and Oda, 2003), ii) high levels of Aβ can induce neuronal hyperexcitability and progressive epilepsy (Minkeviciene et al., 2009), iii) APP transgenic mice exhibit spontaneous seizures more frequently than wild-type mice (Mohajeri et al., 2002; Del Vecchio et al., 2004; Lalonde et al., 2005; Palop et al., 2007; Westmark et al., 2008), iv) prevalence of unprovoked seizures is markedly higher in AD cases than in control populations (Amatniek et al., 2006; Palop and Mucke, 2009; Larner, 2010). Our recent result further demonstrates that kainic acid treatment can markedly enhance the level/processing of APP leading to increased production of Aβ peptide most likely from a subset of astrocytes which are known to become reactive following kainic acid treatment. Additionally, we showed that increased production of Aβ peptide from cultured astrocytes may participate in the kainic acid-induced degeneration of neurons (Kodam et al., 2019). Accompanying Aβ peptide, there is some evidence that tau protein may have a role in the development and/or progression of TLE.

Microtubule associated Tau protein is a soluble, natively unfolded cytoplasmic protein which is involved primarily in the stabilization of microtubules by binding at the interface between tubulin heterodimers (Ren and Sahara, 2013; Wang and Mandelkow, 2016; Tapia-Rojas et al., 2019). In the adult brain, there are six different isoforms of tau, generated by alternative splicing of tau gene located on chromosome 17, encode protein ranging from 352 to 441 amino acids (Fig 1.1). The isoforms generated by splicing exons 2 and 3 lead to the absence or presence of 1- or 2 N-terminal repeats (labelled as 0N, 1N or 2N) and splicing of exon 10 results in tau containing 3- or 4-repeats (labelled as 3R or 4R) of microtubule binding domain. The six different isoforms generated by alternative splicing include: 0N/3R, 0N/4R, 1N/3R, 1N/4R, 2N/3R, and 2N/4R, where "R" represents the number of microtubules binding repeats and "N" corresponds to the number of N-

terminal inserts (Gong et al., 2005; Wang and Mandelkow, 2016; Guo et al., 2017; Tapia-Rojas et al., 2019).

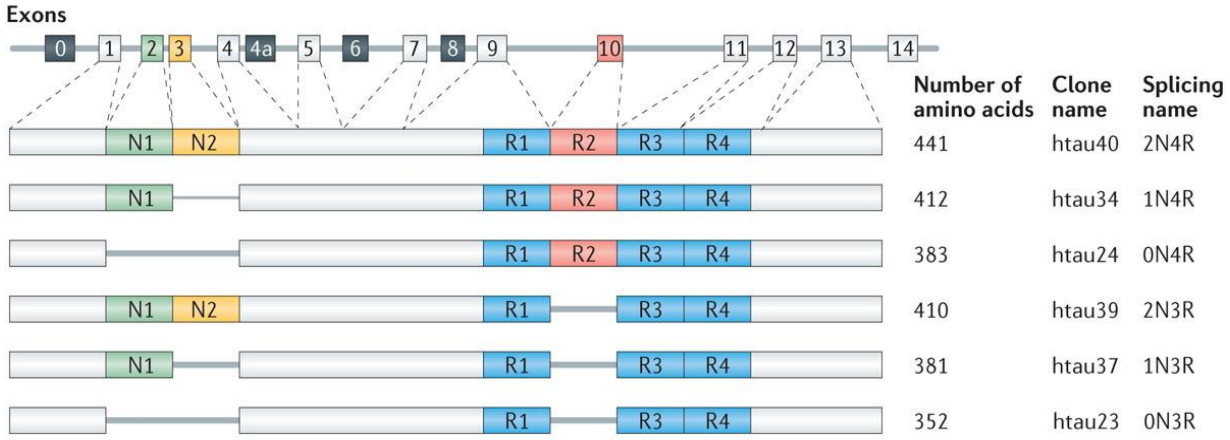

Figure 1.1: Figure showing the MAPT gene and the splice isoforms of tau in the human brain.
From: Wang and Mandelkow, 2016. /PMID: 26631930/ Lic. no: 5274290905121

Under normal condition tau is found mostly in neuronal axons and to a lesser degree in astrocytes and oligodendrocytes. Physiologically, tau binds and stabilizes microtubules regulating axonal transport *via* site-specific phosphorylation-dephosphorylation process mediated by multiple kinases and phosphatases, respectively. However, in pathological conditions, hyperphosphorylation promotes their detachment from microtubules leading to fibrillization into single-straight/paired helical filament (PHFs) and deposition as NFTs in AD brains (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986). In addition to AD, tau has been implicated in a variety of other neurodegenerative diseases collectively termed as "taupathies" such as frontotemporal dementia linked with chromosome 17, Pick disease and supranuclear palsy (Wang and Mandelkow, 2016).

A role for tau in TLE has also been suggested by the results which showed that i) animal models (Crespo-Biel et al., 2007; Tian et al., 2010; Jones et al., 2012; Alves et al., 2019) of TLE as well as human TLE patients (Tai et al., 2016; Gourmaud et al., 2020) exhibit increased levels of tau phosphorylation, ii) knockdown of tau gene ameliorates seizure activity (Holth et al., 2013), whereas tau overexpression induces spontaneous seizures (García-Cabrero et al., 2013) and iii) increase seizure activity observed in transgenic mice overexpressing Aβ peptide occurs in the presence of wild-type tau (Palop et al., 2007; Roberson et al., 2007). Consequently, the reduction of tau can play a major role in disease-modifying treatment for both epilepsy and AD. Despite these findings, very little is known about the significance of tau or its association to degenerating neurons/reactive astrocytes in TLE pathogenesis.

1.6 *TLE and astrocytes*

Astrocytes are star-shaped glial cells that make up the majority of the central nervous system's glial cells (Allen and Barres, 2009). Although known for decades as merely supporting cells or "brain glue", to serve homeostatic functions, including supply of nutrients to neurons and clearance of neuronally released K^+ and glutamate from the extracellular space, astrocytes have recently been shown to play a direct role in synaptic transmission by releasing gliotransmitters such as glutamate, GABA and D-serine (Seifert et al., 2006; Barres, 2008; Foresti et al., 2011). Additionally, astrocytes are found to express almost the same set of ion-channels and receptors as neurons (Verkhratsky and Steinhäuser, 2000; Kettenmann and Steinhäuser, 2004; Seifert and Steinhäuser, 2004). These results not only highlight the importance of astrocytes in normal brain function, but also their potential implications in neurological disorders such as epilepsy, which is accompanied by massive proliferation of glial cells (Seifert et al., 2006; Foresti et al., 2011).

A role for astrocytes in TLE is suggested by data which showed that i) seizure duration correlates with glial density in CA2/CA3 regions of the hippocampus (Spencer et al., 1999), ii) glutamate released from astrocytes contributes to cell death in an animal model of epilepsy (Ding et al., 2007), iii) mice knockout of the astroglial transporter GLT1, responsible for the bulk clearance of extracellular glutamate, exhibit spontaneous seizures and hippocampal pathology resembling TLE (Tanaka et al., 1997) , and iv) a decreased level of glutamine synthase (enzyme that converts glutamate to glutamine) in patients with TLE may result in the accumulation of glutamate in astrocytes and the extracellular space due to slowing of the glutamate-glutamine cycle (Petroff et al., 2002). These data, together with the evidence that changes in astrocytes precede neuronal damage (Kang et al., 2006; Eid et al., 2008; Seifert et al., 2010), provide an underlying basis for a major role for astrocytes in the pathogenesis of epilepsy. Over the years some studies have shown that phosphorylated and/or cleaved-tau can accumulate in a subset of astrocytes in various neurodegenerative diseases, but its association and significance in epilepsy, if any, remains unclear (Kovacs et al., 2017; Leyns and Holtzman, 2017).

1.7 *Heterogeneity of astrocytes*

There are a growing body of recent evidence reveals that astrocytes are highly heterogenous, both morphologically and functionally, in each brain region (i.e., local heterogeneity) as well as across different brain regions (inter-regional heterogeneity). This is evident by the fact that apart from the classical marker glial fibrillary acid protein (GFAP), astrocytes in different brain regions can be identified by using a variety of other markers including vimentin, the glutamate-aspartate transporter GLAST, the calcium binding protein S100β, glutamine synthetase (GS), glutamate transporters such as GLT-1 and aldehyde dehydrogenase 1 family member L1 (Aldh1l1). Although some of these markers are less specific than GFAP, they have been reported to exhibit selectivity

of astrocytic protein expression among different regions of the brain. These data not only suggest the existence of different phenotypic profiles of astrocytes in various brain regions but also raise the possibility that they may mediate different functions in normal and/or pathological conditions (Zhang and Barres, 2010; Liddelow and Barres, 2017; Khakh and Deneen, 2019). However, the molecular mechanisms underlying these changes remain unclear. Recently two different types of reactive astrocytes i.e., A1 and A2 have been proposed in response to different insults (Zamanian et al., 2012; Liddelow et al., 2017; Yun et al., 2018; Matias et al., 2019). In lipopolysaccharide-induced inflammatory scenario, activated microglia triggered a neurotoxic reactive astrocyte phenotype designated as A1 by secreting interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and complement component 1q (C1q). These A1 astrocytes showed loss of normal function and gain of new harmful functions. Conversely, ischemic insult can induce a protective A2 reactive astrocyte which secrete neuroprotective molecules such as thrombospondins (TSPs) and promote central nervous system recovery and repair (Liddelow et al., 2017; Clarke et al., 2018; Yun et al., 2018). There are growing consensus that the A1 or A2 (Good *vs* Bad), disease associated astrocytes (DAA) or other generalizing nomenclature for reactive astrocytes may not be applicable to a variety of disorders and is controversial (Habib et al., 2020; Escartin et al., 2021). Furthermore, it remains to be seen whether different subsets of astrocytes are differentially activated in kainic acid model of TLE and their association, if any, with tau protein and/or disease pathogenesis. Thus, the present study is designed to determine the level/expression of different subset of astrocyte markers and their association to tau in the kainic acid model of mTLE.

1.8 *Hypothesis and objectives*

Based on the introduction and review of the literature, we want to hypothesize that astrocytes are differentially activated following kainic acid administration and there is altered levels/expression

of tau protein in a subset of reactive astrocytes which may have an important role in the progression of disease pathology in animal models of experimental TLE. To address this hypothesis our main objectives are:

1) To characterize the level/expression of different subset of astrocyte markers following kainic acid treatment.

2) To define if kainic acid treatment can induce phosphorylation and/or cleavage of tau protein *in vivo* and their association to the progression of seizure and/loss of neurons.

2. METHODS

2.1 *Materials*

Kainic Acid monohydrate (≥ 99% purity), Sodium dodecyl sulfate (SDS), bovine serum albumin, Trizma base, 3,3'-Diaminobenzidine (DAB) and tween-20 were obtained from Sigma-Aldrich (Oakville, ON, Canada). The bicinchoninic acid (BCA) protein assay kit, triton-X, fluorescently conjugated secondary antibodies, enhanced chemiluminescence (ECL) kit and paraformaldehyde (PFA) were from Thermo Fisher Scientific (Montreal, QC, Canada), whereas acrylamide for in-house gel preparation, glycine and horseradish peroxidase (HRP)-conjugated secondary antibodies was from Bio-rad (Hercules, CA, USA), immobilon-P PVDF membrane from Merck Millipore Ltd (Cork, Ireland), Pro-long gold antifade mounting media from Invitrogen (Eugene, OR, USA), Lambda Protein Phosphatase (Lambda PP) was from New England Biolabs Ltd and Fluoro-Jade C was from Chemicon Int., (Temecula, CA, USA). Fast Red Substrate kit and Goat Anti-Mouse IgG H&L (Alkaline Phosphatase) was purchased from Abcam (MA, USA). Sources of all primary antibodies and their dilutions used in this study are listed in **Table-1**. All other chemicals were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Thermo Fisher (Waltham, MA, USA).

2.2 *Animals and kainic acid treatment*

Adult male Sprague-Dawley rats (170-200g) obtained from Charles River (St. Constant, Qc, Canada) were used in the study. All animals, maintained on a 12-hour light/dark cycle, were housed with access to food and water *ad libitum*. The experiments were performed in accordance with the Institutional and Canadian Council on Animal Care guidelines. Adult rats were injected intraperitoneally with either kainic acid dissolved in normal saline (12 mg/kg) or equal volume

(0.2 - 0.25 ml) normal saline as described earlier (Kar et al., 1997; Kodam et al., 2019). After treatment, a subset of control and kainic acid-treated rats were euthanized at 12hr, 2 days and 12 days by decapitation and their hippocampi were dissected out of the brain and frozen immediately for biochemical assays. For anatomical study, another subset of control and kainic acid-treated rats were transcardially perfused first with phosphate-buffered saline (0.01M PBS, pH 7.4), followed by 4% paraformaldehyde (PFA). Brains were dissected out, post fixed in 4% PFA, cryoprotected in 30% PBS-sucrose, sectioned (20-40μm) on a cryostat and then processed using a free-floating manner as described earlier (Kodam et al., 2019).

2.3 *Western blotting*

Hippocampal tissues from control and kainic acid-treated rats from various time points (4-6 animals/group) were homogenized in ice-cold radioimmunoprecipitation assay (RIPA)-lysis buffer and then centrifuged at 10,000rpm for 10minutes at 4°C. The supernatant was collected, and protein content was determined using a BCA kit and then normalized to a common working concentration with RIPA buffer. An equal amount of protein was boiled in 6 volumes of SDS sample buffer for 10minutes and separated on 10%, 12% or 7-17% gel and transferred to PVDF membrane. The membrane was blocked in 5% skim milk in TBST [10 mm Tris-HCL (pH 8.0), 150 mm NaCl, and 0.2% Tween-20] for 1hour at room temperature and incubated overnight at 4°C with anti-GFAP, anti-Vimentin, anti-GS, anti-S100β, anti-Aldh1l1, anti-C3, anti-S100A10, anti-Iba1, anti-Tau5, anti-Tau1, anti-Caspase Cleaved-Tau (D421) and anti-Phospho-tau (AT-270 and anti-PHF1) antibodies at dilutions listed in Table-1. After incubation, membranes were washed with TBST for 10minutes, 3 times each and then incubated with HRP-conjugated secondary antibodies for 1hour at room temperature. The membranes were finally washed with TBST for 30minutes and developed using an ECL detection kit. Membranes were subsequently re-probed

with anti-β-actin to monitor protein loading. The immunoreactive proteins from all blots were subsequently quantified using image-J program as described earlier (Kodam et al., 2019; Ourdev et al., 2019).

2.4 *Fluoro-Jade C staining*

Sections from control and kainic acid-treated rat brains were processed sequentially with 70% alcohol, distilled water and 0.06% potassium permanganate solution. Subsequently, the sections were incubated with 0.0001% Fluoro-Jade C in 0.1% acetic acid for 10minutes at room temperature. Stained sections were washed thoroughly in distilled water, processed, and then mounted and examined using a Nikon eclipse-90i fluorescence microscope as described earlier (Kodam et al., 2019).

2.5 *Enzyme-linked Immunohistochemistry*

Brain sections from control and kainic acid-treated rats (3-5 animals/group) were processed for immunohistochemical labelling as described earlier (Banerjee et al., 2015). In brief, brain sections were first washed in PBS (0.01M, pH 7.4), treated with 1% hydrogen peroxide for 30 minutes, blocked with normal goat serum (10%) and then incubated overnight at 4°C temperature with anti-GFAP and anti-S100A10 antibodies with dilutions as indicated in Table 1. The sections were exposed to appropriate secondary antibodies, HRP-based for S100A10 and H&L (Alkaline Phosphatase) based for GFAP for 1-2 hours at room temperature, washed using PBS. Finally, for S100A10 and GFAP, the sections were developed using either the glucose-oxidase-nickel enhancement method the DAB or the Fast Red detection system (AP). Both primary antibodies were applied for co-labelled, and then DAB was used to develop S100A10, followed by Fast Red to develop GFAP the next day, resulting in a co-stained picture of the hippocampus and then

mounted with Prolong gold antifade reagent. Immunostained sections were then examined/imaged using HAMAMATSU Nanozoomer 2.0-RS. Three to five double labelled sections from control (n = 3-5) and kainic acid-treated (n = 3-5) rats were consequently used to quantify double labelled glial cells using the Image-J program.

2.6 *Immunofluorescence staining*

Brain sections from control and kainic acid-treated rats (3-5 animals/group) were processed for double immunofluorescence staining as described earlier (Kodam et al., 2019). In brief, brain sections were first washed in PBS, treated with 0.02% triton-X, and then labelled sequentially with anti-S100β, anti-Vimentin, anti-GS, anti-Aldh1l1 and anti-cleaved tau antisera followed by anti-GFAP or Iba1 antisera at dilutions listed in Table-1. The primary antibodies were labelled with either Texas Red- or FITC-conjugated secondary antibodies (1:500) for 1-2 hours at room temperature, washed and then mounted with Prolong gold antifade reagent. Immunostained sections were examined and photographed using a Nikon eclipse-90i fluorescence microscope. Three to five double labelled sections from control (n = 3-5) and kainic acid-treated (n = 3-5) rats were used to quantify double labelled glial cells using the Image-J program (Fiji) as described earlier (Banerjee et al., 2015).

2.7 *Data analysis*

Data are expressed as Mean ± SEM. Comparisons between groups were performed using ANNOVA utilizing Tukey's post-hoc multiple comparisons test. A *p*-value of less than 0.05 was accepted as statistically significant. All statistical analysis was performed using GraphPad Prism software (GraphPad Inc., CA, USA). ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

2.8 *Table 1: Details of primary antibodies used in the study*

S/N	Antibody Name	Host	Type	Dilution		Source
				WB	IHC/IFC	
1.	Anti-GFAP	Mouse	Monoclonal	1:1000	1:1000	Abcam
2.	Anti-GFAP	Chicken	Polyclonal	NA	1:1000	Abcam
3.	Anti-Vimentin	Chicken	Polyclonal	1:1000	1:500	Abcam
4.	Anti-Glutamine Synthetase (GS)	Rabbit	Polyclonal	1:500	1:500	Abcam
5.	Anti-S100β	Rabbit	Polyclonal	1:500	1:500	Abcam
6.	Anti-Aldh1l1	Rabbit	Polyclonal	1:500	1:1000	Abcam
7.	Anti-C3	Mouse	Monoclonal	1:500	1:200	R&D System
8.	Anti-S100A10	Rabbit	Polyclonal	1:500	1:500	Abcam
9.	Anti-Iba1	Rabbit	Monoclonal	1:500	1:1000	Wako
10.	Anti Tau-1 (Clone PC1C6)	Mouse	Monoclonal	1:500	NA	Millipore
11.	Anti-Tau-5 (Clone tau 5)	Mouse	Monoclonal	1:500	NA	Millipore
12.	Anti- AT270	Mouse	Monoclonal	1:500	NA	Sigma
13.	Anti-PHF 1	Mouse	Monoclonal	1:500	NA	Peter Davies
14.	Anti-Caspase Cleaved Tau (Asp421)	Mouse	Monoclonal	1:500	1:200	Millipore
15.	β - actin	Mouse	Monoclonal	1:5000	NA	Sigma
16.	Tubulin	Mouse	Monoclonal	1:5000	NA	Abcam

NA, not applicable.

3. RESULTS

3.1 ***Neuronal degeneration and glial cell activation in kainic acid-treated rat***: In keeping with our earlier results (Banerjee et al., 2015; Kodam et al., 2019), systemic administration of kainic acid induced seizures characterized by "wet-dog" shakes, rearing and falling, facial and forelimb clonus that lasted about 8-10hrs. The animals were monitored closely for about 6-8hrs following injection of kainic acid and then every 24hrs until the end of experiment. Over the course of 12days of experiment, majority of the animals that survived the initial attack exhibited spontaneous recurrent limbic seizures with no remission following few days of seizure-free period. These behavioral changes are accompanied by degeneration of neurons, as apparent by Fluoro-Jade C labeling (Fig. 3.1A, B), as well as hypertrophy of the glial cells immunolabelled with GFAP and Iba1 in the hippocampus of the brain (Fig. 3.1C-F). The degeneration of neurons, as reported earlier (Banerjee et al., 2015; Kodam et al., 2019), progressed in a time-dependent manner in the CA1-CA3 pyramidal neurons, whereas the granular cells are relatively spared. Accompanying the hypertrophy of astrocytes and microglia, the steady-state levels of GFAP and Iba1 increased significantly at 12hrs post-treatment and then elevated gradually over 12days of experimental time-course (Fig. 3.1G and H). The gradual degeneration of neurons and activation of glial cells were seen in the hippocampus of both hemispheres. Although kainic acid treatment is known to influence degeneration of neurons and alterations of various neurochemical parameters in other regions of the brain such as amygdala, cortex and thalamus, the present study focussed on the hippocampus – which plays a critical role in TLE pathogenesis.

3.2 ***Effects of kainic acid treatment on different subsets of astrocytes***: Given the diversity of astrocytes which are present across brain regions (Anderson et al., 2014), we intend to evaluate

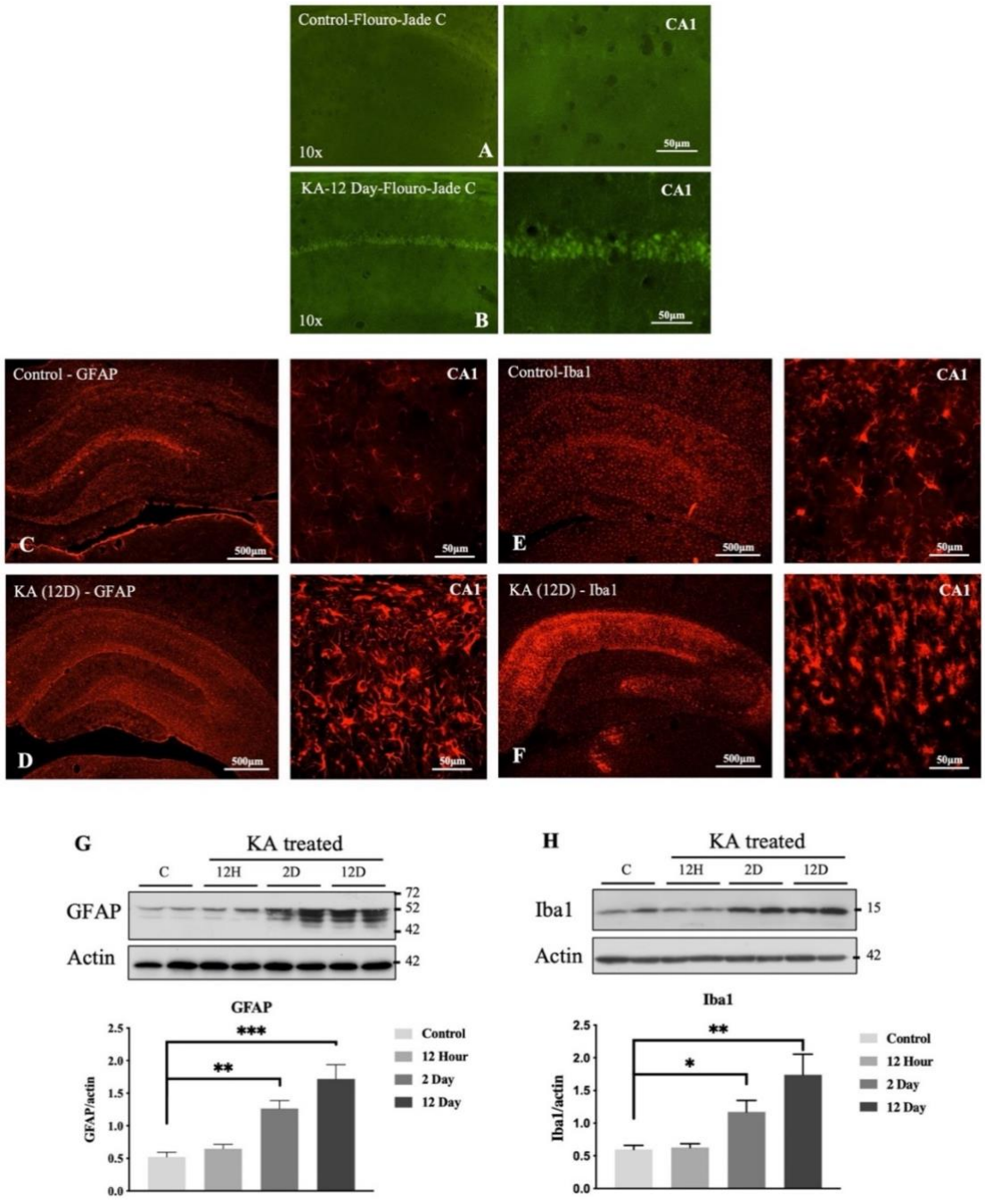

Figure 3.1: Fig (A & B) show control and 12day kainic acid-treated rats labelled with Fluoro-Jade C. Fig (C & D) show the change in expression of astrocytes (GFAP) and (E & F) shows the change in the expression level of microglia (Iba1) in the hippocampus from control to kainic acid-12D treated rats. Histogram and representative immunoblot (G & H) show the level of astrocytes (GFAP) and microglia (Iba1) in the hippocampus of kainic acid-treated rats. Blots were re-probed with β-actin to monitor protein loading. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

if kainic acid treatment can differentially alter the level and expression of various astrocyte markers such as Vimentin, S100β, Aldh1l1 and GS in relation to classical astrocyte marker GFAP in the hippocampal region of the brain.

Vimentin is a type III intermediate filament that maintains cell integrity, and is involved in cell migration, motility and adhesion. It is known to be expressed in a subset of astrocytes as well as endothelial cells. There is evidence that vimentin in the adult brain is apparent in fibrous astrocytes and is known to be upregulated following seizures and traumatic CNS lesions (Schnitzer et al., 1981; Ridet et al., 1995; Stringer, 1996; Aronica et al., 2000). Our results revealed that steady state levels of vimentin, as observed with GFAP, did not alter at 12hr but increased significantly from 2day onwards in the hippocampus of kainic acid-treated rats compared to controls (Figs. 3.1G and 3.2A). At the cellular level, very few vimentin-labelled astrocytes, present in control hippocampus, are distributed evenly in the CA1-CA3 subfields as well as in the dentate gyrus. It is of interest to note that very few GFAP-labelled astrocytes express vimentin in the hippocampus under normal condition. Following kainic acid treatment, vimentin-positive astrocytes increased markedly, especially in the CA1-CA3 subfields of the hippocampus. The number of GFAP-labelled astrocytes expressing vimentin, as evident by double immunolabelling, increased from 1% in control group to 10% in 12day post-treated kainic acid rat hippocampus (Fig. 3.2B-H).

The protein S100β is a calcium-binding cytoplasmic protein found in a wide range of cells, including a subset of astrocytes. It has been shown that S100β is expressed by a subtype of mature astrocytes that ensheath blood vessels under normal condition. It is known to be involved in the regulation of a number of cellular processes including cell cycle progression and differentiation and suggested to have a role in epilepsy (Griffin et al., 1995; Ridet et al., 1995; Girardi et al., 2004; Lu et al., 2010). Our western blot data revealed that S100β did not alter at 12hr or 2day but

increased markedly following 12day post-treatment in the hippocampus of kainic acid-treated rats compared to saline-treated controls (Figs. 3.3A). At the cellular level, S100β-immunoreactivity is evident throughout the hippocampus of control rats and a subset of them are co-localized with GFAP-labelled astrocytes. Following kainic acid treatment, the number of S100β-labelled astrocytes increased markedly in the CA1-CA3 subfields of the hippocampus. In parallel, the number of S100β-labelled astrocytes expressing GFAP increased from 15% to 17% in the hippocampus of 12day kainic acid-treated rats (Fig. 3.3B-H).

Aldh1l1, also named as 10-formyltetrahydrofolate dehydrogenase, is a folate enzyme that converts 10-formyltetrahydroifolate to tetrahydrofolate. It is crucial in several biochemical reactions including nucleotide biosynthesis and is involved in cell division and growth. It is found to be expressed in a subset of mature and immature astrocytes as well as in reactive astrocytes following injury (Anthony and Heintz, 2007; John Lin et al., 2017; Westergard and Rothstein, 2020). It is evident from our immunoblot analysis that Aldh1l1 level in the hippocampus increased significantly at 12day, but not at 12hr or 2day, following treatment with kainic acid compared to controls (Figs. 3.4A). In control hippocampus, Aldh1l1-positive astrocytes were apparent mostly in the CA1-CA3 subfield and to some extent in the dentate gyrus; a subset of which is found to express GFAP. Interestingly, kainic acid treatment not only increased the number of Aldh1l1-labelled astrocytes but also enhanced the number of astrocytes expressing Aldh1l1 and GFAP from 9% to 16% in the hippocampus after 12day post-treatment (Fig. 3.4B-H).

The enzyme GS is involved in a variety of brain functions including the metabolic regulation of glutamate (glutamate-glutamine cycle), detoxification of ammonia, recycling of neurotransmitter and terminating neurotransmitter signals. In the brain GS is evident in a subpopulation of astrocytes and is known to be involved in various pathological conditions including TLE

(Norenberg and Martinez-Hernandez, 1979; Papageorgiou et al., 2011; Anlauf and Derouiche, 2013; Sandhu et al., 2021). The steady state levels of GS, unlike GFAP, vimentin, S100β and Aldh1l1, did not alter as a function of time in the hippocampus of kainic acid-treated rats (Figs. 3.5A). In control hippocampus, a subset of GS-positive astrocytes is found to express GFAP. Interestingly, kainic acid administration did not alter the number of GS-positive astrocytes or its localization with GFAP-labelled astrocytes after 12day post-treatment compared to control rats (Fig. 3.5B-H). This result suggested the differential increase in different astrocytic markers by kainic acid.

3.3 ***Effects of kainic acid treatment on C3 positive and S100A10 positive subsets of astrocytes***: Previous studies have tried to categorize astrocytes into neurotoxic "A1" or neuroprotective "A2" following different insults; A1 astrocytes are shown to exhibit increased expression of complement component C3, whereas A2 like astrocytes has higher level of calcium binding S100A10 protein (Zamanian et al., 2012; Liddelow et al., 2017). To determine if kainic acid treatment can differentially alter these astrocytic expressing proteins, we performed western blotting as well as immunohistochemistry using C3 and S100A10 markers as reported in earlier studies (Liddelow et al., 2017; Yun et al., 2018; Hartmann et al., 2019). Our Western blot analysis clearly show a significant progressive increase in the levels of C3 in the hippocampus of 2 and 12day kainic acid post-treated rats compared to controls (Fig. 3.6A). At the cellular level, C3-immunoreacitivity in control hippocampus is apparent in few scattered cells, some of which exhibit co-labelled with GFAP. Kainic acid treatment not only increase the number of C3-labelled astrocytes but also enhanced the number of astrocytes expressing C3 and GFAP from 4 % to 17 % in the hippocampus after 12day post-treatment (Fig. 3.6B-H). Accompanying C3 level/immunoreactivity, a significant increase in the level of S100A10 was evident in the hippocampus of kainic acid-treated rats at all

time points (i.e., 12hrs, 2day and 12day) compared to controls (Fig. 3.7A). The increase in S100A10 level was observed from 12hrs onward following kainic acid treatment. Consistent with our western blot data, S100A10 immunoreactivity is evident in the hippocampus and a subset of GFAP-labelled astrocytes express immunoreactive S100A10. Following kainic acid treatment, not only the number of S100A10 labelled astrocytes but their co-labelled with GFAP also markedly increase from 31% to 38% in the hippocampus of 12day after treatment (Fig. 3.7B-H). These results, taken together, further suggest that kainic acid treatment differentially affects various subpopulation of astrocytes in the adult rat hippocampus.

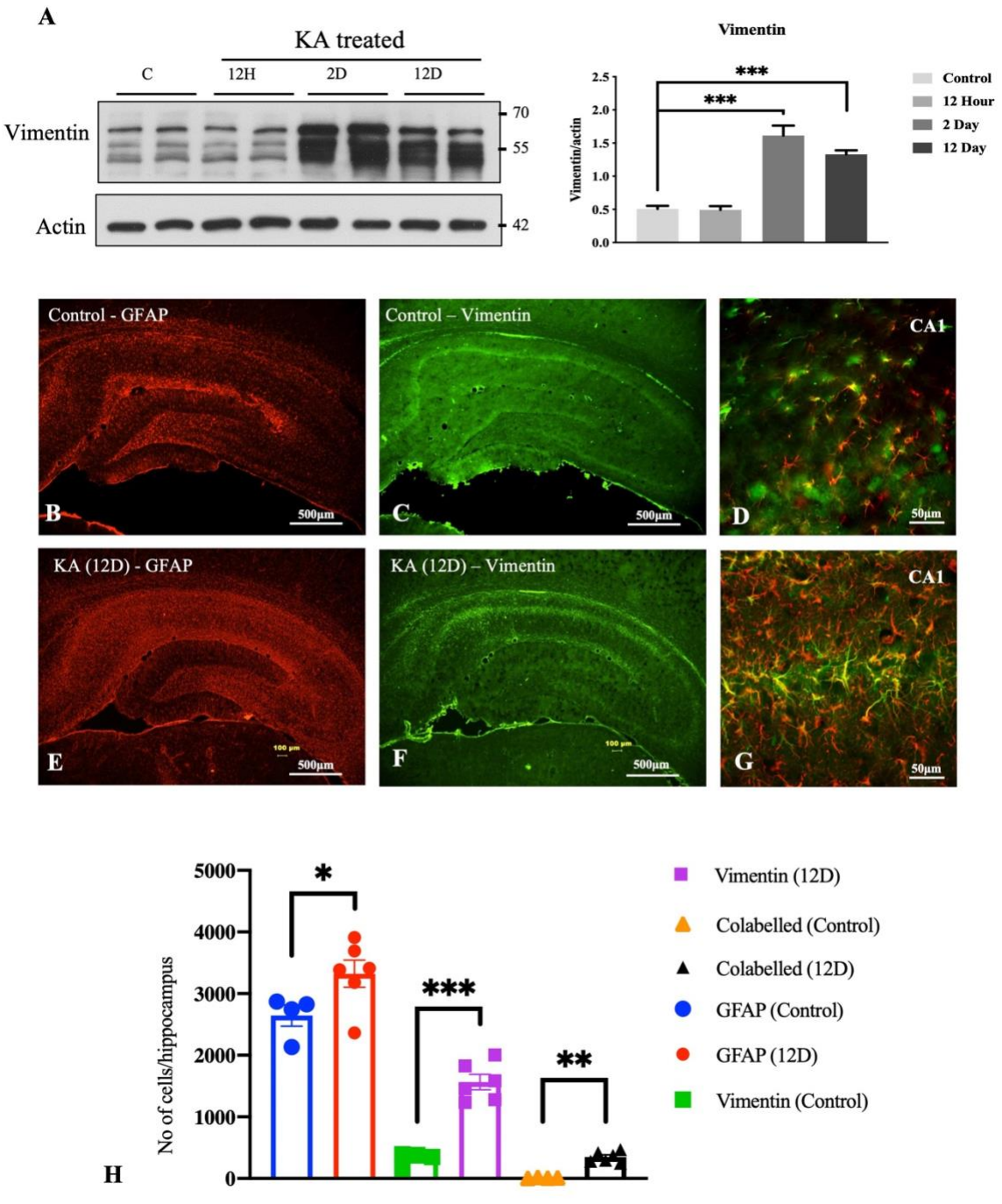

Figure 3.2: Histogram and representative immunoblots (A) showing the change in level of astrocytic marker (Vimentin) in the hippocampus of kainic acid-treated rats. Fig (B-G) shows the level/expression of astrocytes (GFAP and Vimentin) in the hippocampus of control to kainic acid-12D treated rats. Fig (H) is the quantitative data representing the differential expression of Vimentin when co-stained with GFAP. Blots were re-probed with β-actin to monitor protein loading. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

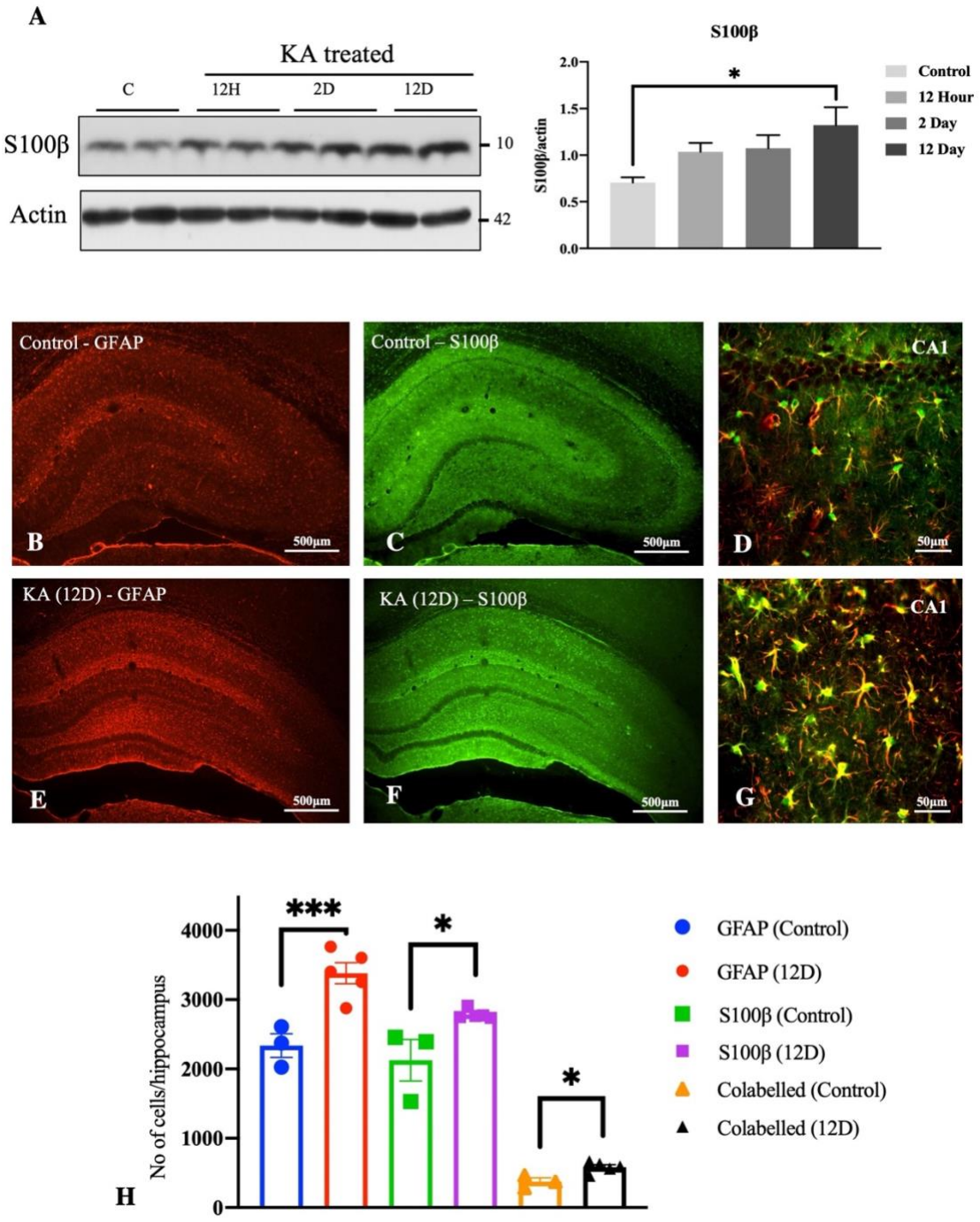

Figure 3.3: Histogram and representative immunoblots (A) showing the change in level of astrocytic marker (S100β) in the hippocampus of kainic acid-treated rats. Fig (B-G) shows the level/expression of astrocytes (GFAP and S100β) in the hippocampus from control to kainic acid-12D treated rats. Fig (H) is the quantitative data representing the differential expression of S100β when co-stained with GFAP. Blots were re-probed with b-actin to monitor protein loading. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

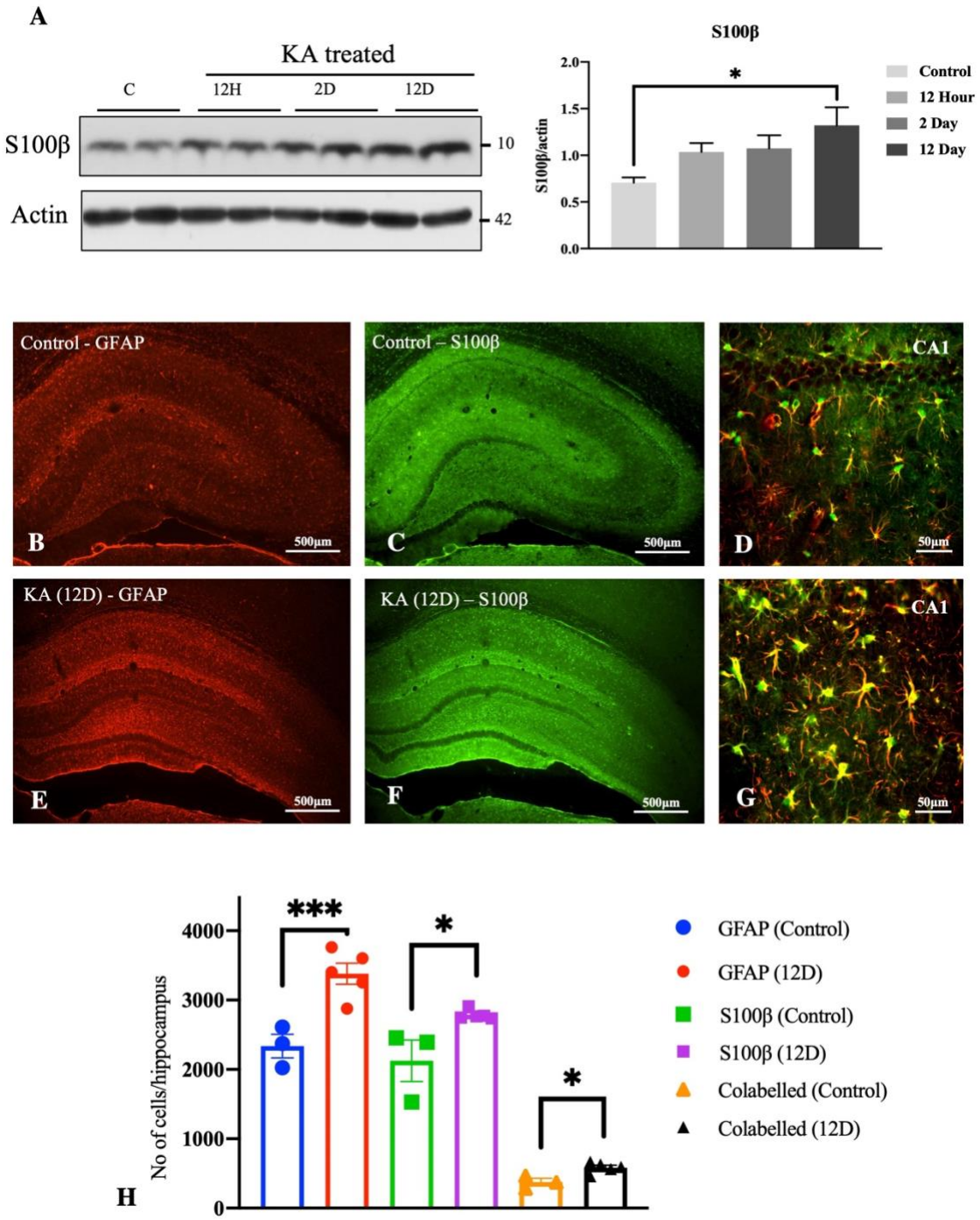

Figure 3.4: Histogram and representative immunoblots (A) showing the change in level of astrocytic marker (Aldh1l1) in the hippocampus of kainic acid-treated rats. Fig (B-G) shows the level/expression of astrocytes (GFAP and Aldh1l1) in the hippocampus from control to kainic acid-12D treated rats. Fig (H) is the quantitative data representing the differential expression of Aldh1l1 when co-stained with GFAP. Blots were re-probed with β-actin to monitor protein loading. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

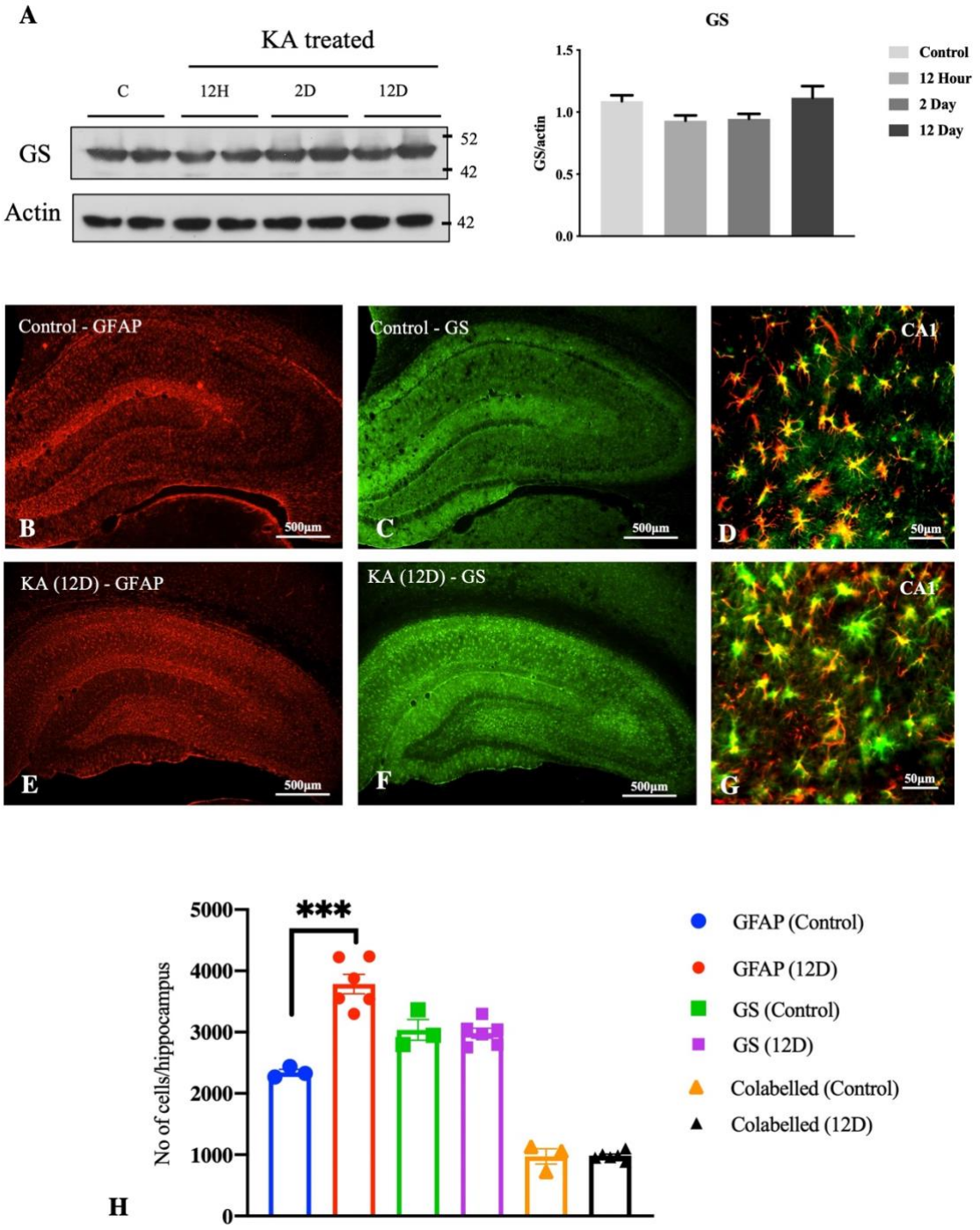

Figure 3.5: Histogram and representative immunoblots (A) showing the change in level of astrocytic marker (GS) in the hippocampus of kainic acid-treated rats. Fig (B-G) shows the level/expression of astrocytes (GFAP and GS) in the hippocampus of from control to kainic acid-12D treated rats. Fig (H) is the quantitative data representing the differential expression of GS when co-stained with GFAP. Blots were re-probed with β-actin to monitor protein loading. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

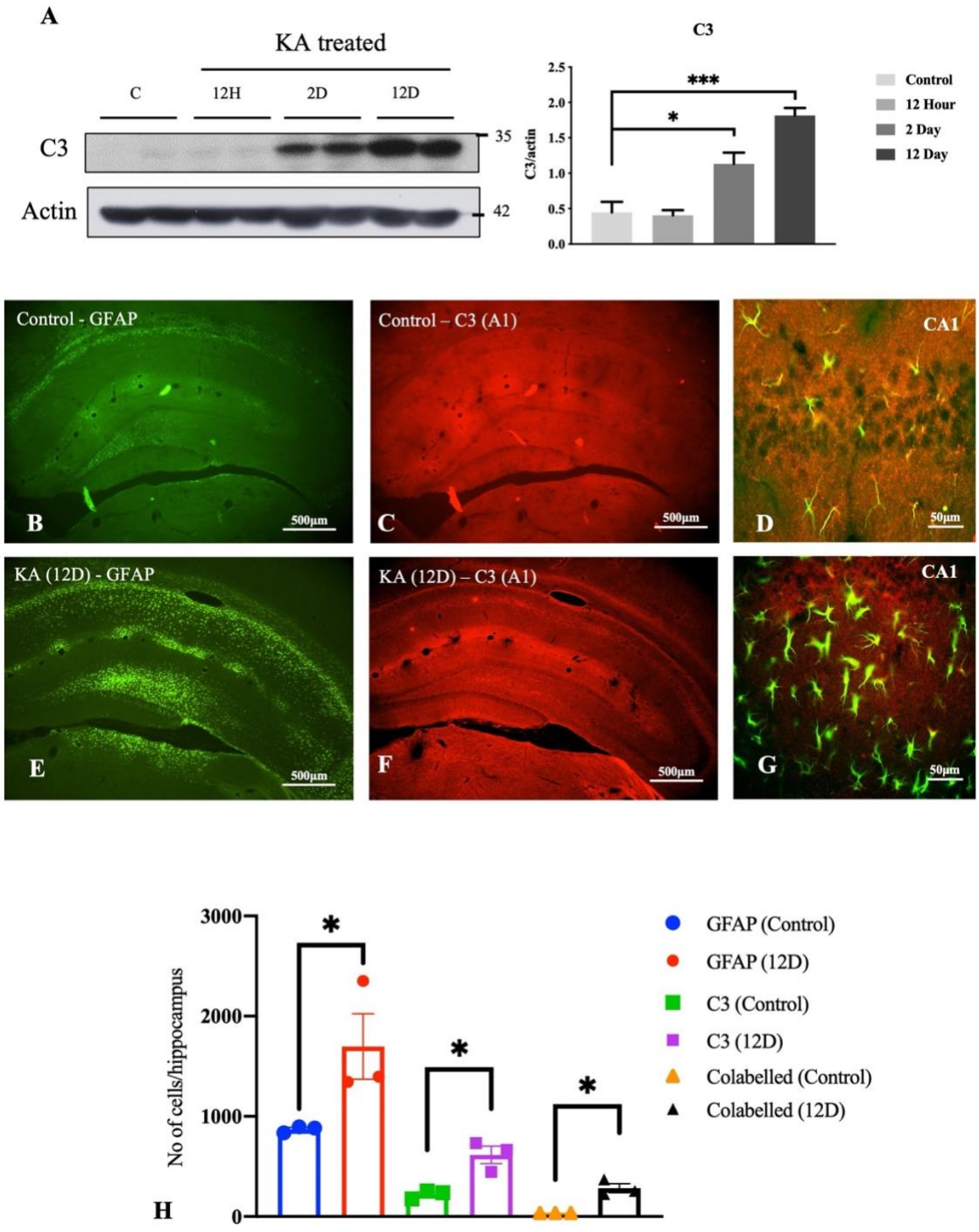

Figure 3.6: Histogram and representative immunoblots (A) showing the change in level of Complement component (C3) in the hippocampus of kainic acid-treated rats. Fig (B-G) shows the level/expression of astrocytes (GFAP and C3) in the hippocampus from control to kainic acid-12D treated rats. Fig (H) is the quantitative data representing the differential expression of C3 when co-stained with GFAP. Blots were re-probed with β-actin to monitor protein loading. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

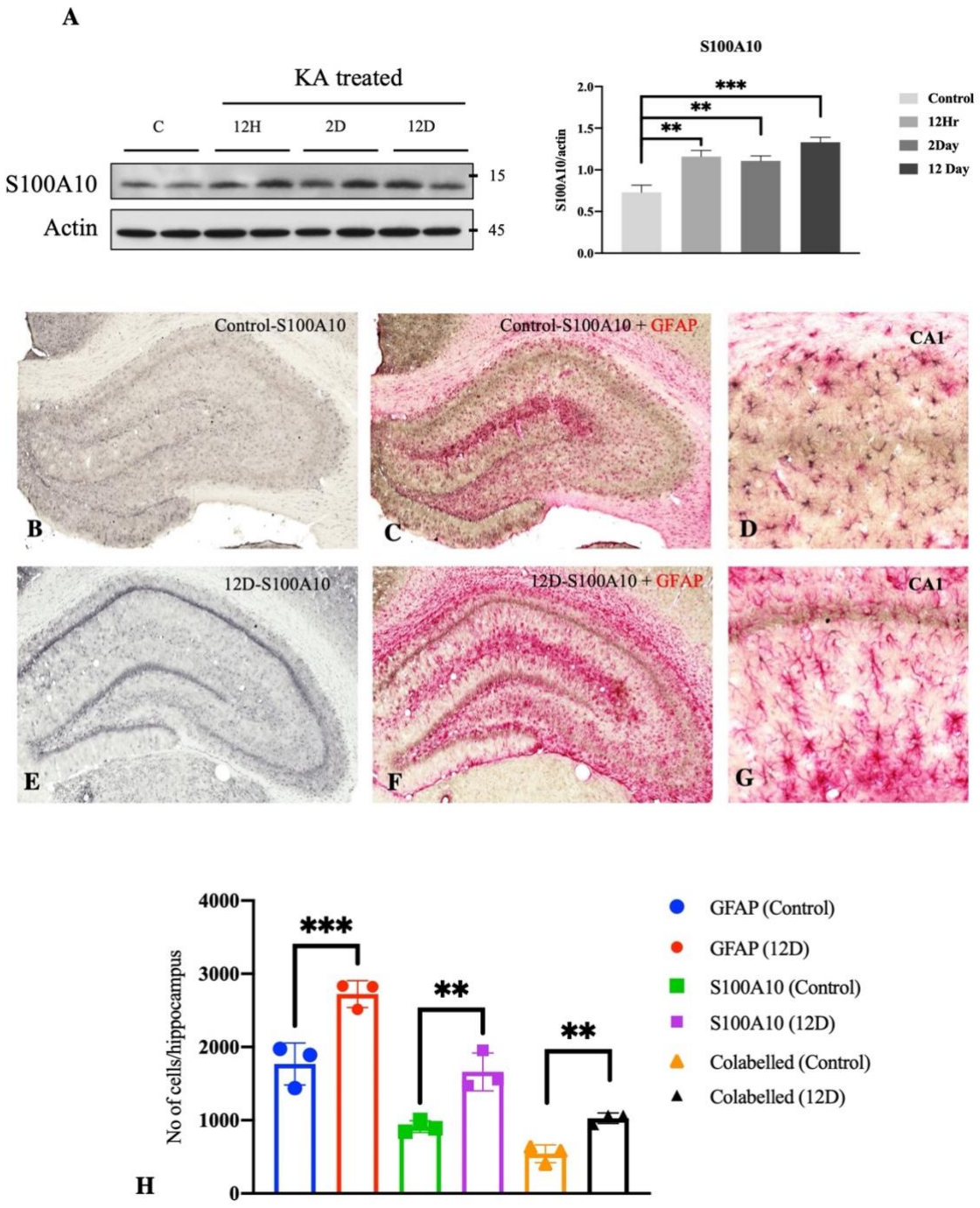

Figure 3.7: Histogram and representative immunoblots (A) showing the change in level of astrocytic marker (S100A10) in the hippocampus of kainic acid-treated rats. Fig (B-G) shows the level/expression of astrocytes (GFAP and S100A10) in the hippocampus of from control to kainic acid-12D treated rats. Fig (H) is the quantitative data representing the differential expression of S100A10 when co-stained with GFAP. Blots were re-probed with β-actin to monitor protein loading. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.4 ***Effects of kainic acid treatment on tau protein***: Previous studies have shown that microtubule associated tau protein plays a critical role in the pathogenesis of epilepsy. This is supported by the evidence that TLE patients, who had epilepsy surgery, exhibited hyperphosphorylated tau pathology in the form of neurofibrillary threads and neurofibrillary tangles in the resected epileptogenic temporal cortex (Tai et al., 2016). Additionally, reducing tau expression has been shown to protect mice carrying AD-related mutations with enhanced Aβ pathology from pharmacologically induced seizures, spontaneous non-convulsive epileptiform activity and early mortality (Palop et al., 2007; Roberson et al., 2007), whereas tau overexpression has been shown to induce neuronal hyperexcitability and seizures (García-Cabrero et al., 2013). Accompanying studies of genetically modified animal models, kainic acid-induced seizure, associated with oxidative stress, inflammatory response and excitotoxicity, has been shown to enhanced the levels of phospho-tau under certain conditions depending on the route/dose of kainic acid administration (Metcalfe and Figueiredo-Pereira, 2010; Leyns and Holtzman, 2017; Didonna, 2020). However, at present, the relationships between the seizure-induced tau phosphorylation and its association to cell types or degeneration of neurons remains unclear. We have recently reported that systemic administration of kainic acid can increase the level/processing of APP and its processing enzymes in activated astrocytes leading to enhanced production of Aβ-related peptides which can contribute to the degeneration of neurons (Kodam et al., 2019). Given the close functional interrelationship between Aβ peptide and tau in a variety of experimental paradigms as well as disease pathology (Revett et al., 2013; Selkoe and Hardy, 2016), we wanted to evaluate the level and/or expression of tau in the rat hippocampus following systemic administration of kainic acid.

To determine the levels of phospho-tau, we first analysed hippocampal samples from control and kainic acid-treated rats from different time-points using various total and phospho-tau antibodies

(12hrs, 2 and 12days) by western blotting. Our results showed that levels of total tau, evaluated using Tau-1 and Tau-5 antibodies, transiently increased significantly (i.e., 12hrs post-treatment) and then decreased (i.e., 2days and 12days post-treatment) in kainic acid-treated rats compared to control rats (Fig. 3.8A and B). Accompanying total-tau, the levels of phospho-tau, determined using AT-270 (Thr^{181}) and PHF1 (Ser^{396}/Ser^{404}) antibodies, also increased at 12hrs and then decreased at 2days and 12days after kainic acid treatment (Fig. 3.9A and B). To validate the transient increase in the phosphorylation of tau protein in kainic acid-treated rats, hippocampal samples were exposed to dephosphorylating enzyme Lambda PP which releases phosphate groups from phosphorylated serine, threonine, and tyrosine residues in proteins and then processed by western blotting using PHF-1 phospho-tau antibody. As expected, enzymatic treatment decreased the phospho-tau level but didn't affect the level of the total tau (detected using Tau-5 antibody) when compared with untreated sample (Fig. 3.8C), thus substantiating our results.

Accompanying phosphorylation, tau protein is known to undergo a variety of other post-translational modifications such as acetylation, methylation, glycation, ubiquitylation, glycosylation and truncation that can influence the formation of neurofibrillary tangles and/or disease pathology. Evidence suggests that tau can be cleaved by calpain as well as caspase leading to the formation of cleaved tau which can play a role in the degeneration of neurons following injury as well as in disease pathology (Gamblin et al., 2003; De Calignon et al., 2010; Hanger and Wray, 2010). The enzyme caspase cleaves tau at Asp^{421} leading to the generation of a short C-terminal peptide and a long truncated NH_2 terminal fragment (Δ421) (Hanger and Wray, 2010; Biundo et al., 2017). Since kainic acid treatment induces activation of caspase and inhibition of caspase activity can attenuate neuronal damage (Faherty et al., 1999; Henshall et al., 2000; Kondratyev and Gale, 2000), we wanted to evaluate by immunoblotting using monoclonal

antibody against D^{421} cleaved tau peptide (Δ421), if the level of cleaved tau is altered following systemic administration of kainic acid. Interestingly, our results showed a gradual increase in the level of cleaved tau in the hippocampus of kainic acid-treated rats which reached significance at 12day post-treatment compared to saline-treated controls (Fig. 3.9C). At the cellular level, cleaved-tau-immunoreactivity is not evident in any specific regions of the hippocampus of control rats but markedly increase in a subset of glial cells. Our double labelling studies further revealed that a subset of GFAP-labelled astrocytes, but not Iba1-labelled microglia, express immunoreactive cleaved-tau in the hippocampus of kainic acid-treated rats (Figs. 3.10 and 3.11). Taken together these results suggest that tau may play an important role in the mechanism of TLE and the cleaved tau fragment of tau may have a role in disease progression.

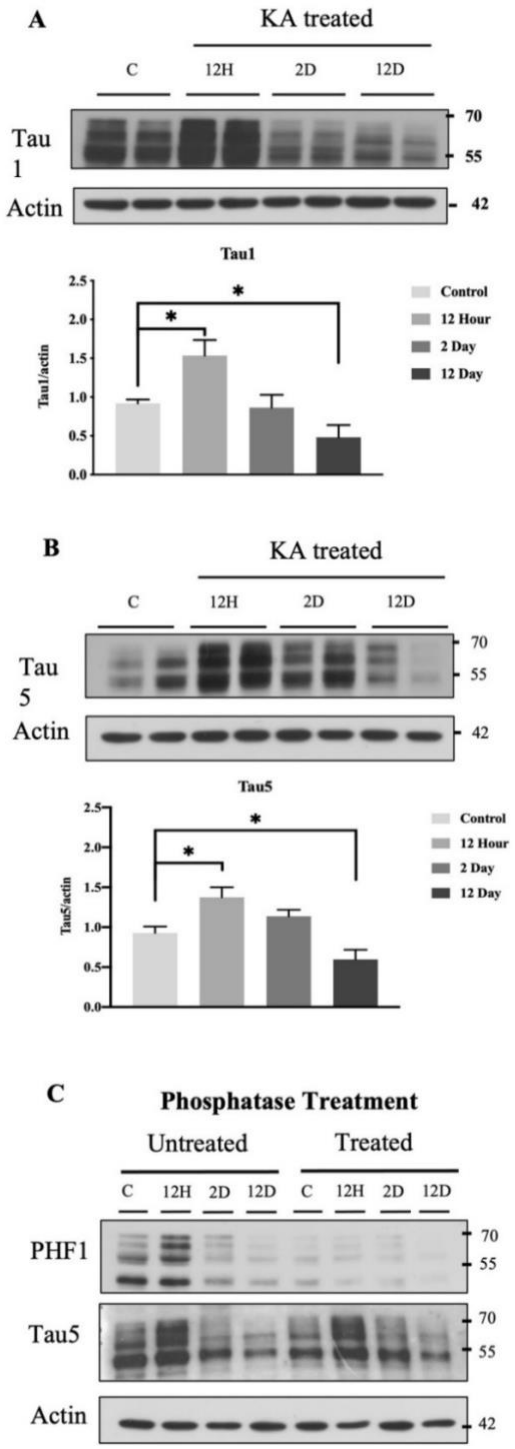

Figure 3.8: Histogram and representative immunoblots (Fig. A & B) showing the level/expression of total tau (Tau1 and Tau5) and in the hippocampus of kainic acid-treated rats. Fig (C) shows the expression of PHF1 and Tau5 after phosphatase enzymatic treatment. Blots were re-probed with β-actin to monitor protein loading.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

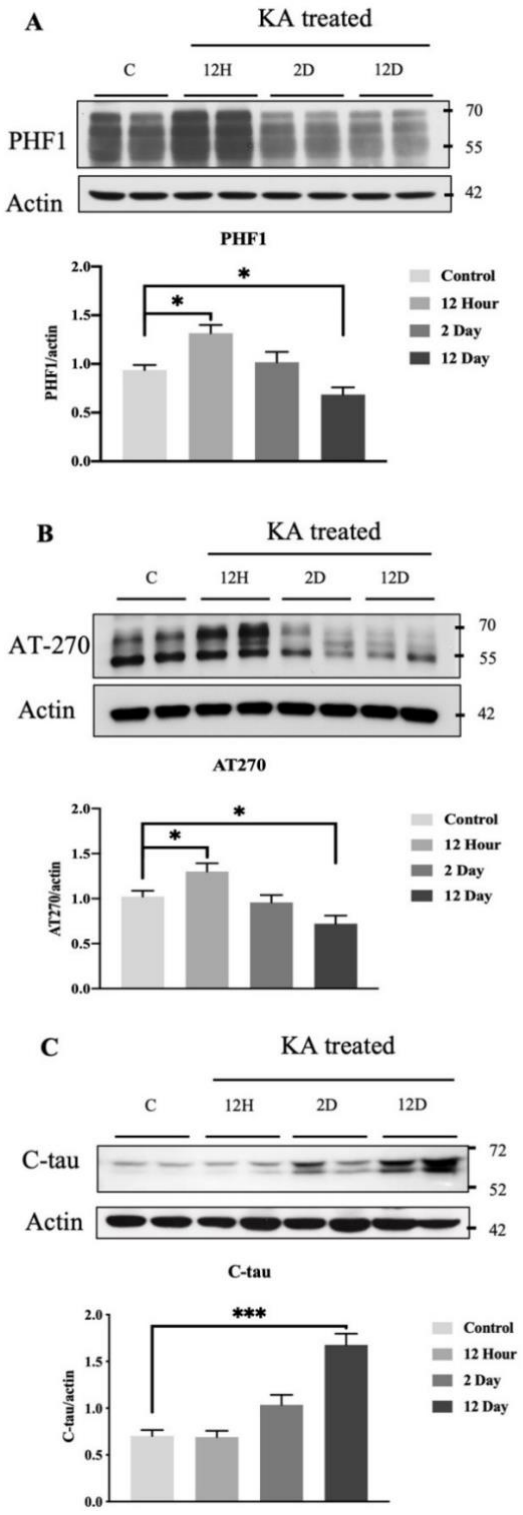

Figure 3.9: Histogram and representative immunoblots (Fig. A & B) showing the level/expression of phospho-tau (PHF1 and AT-270) in the hippocampus of kainic acid-treated rats. Fig (C) shows the levels/expression of cleaved tau. Blots were re-probed with β-actin to monitor protein loading. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

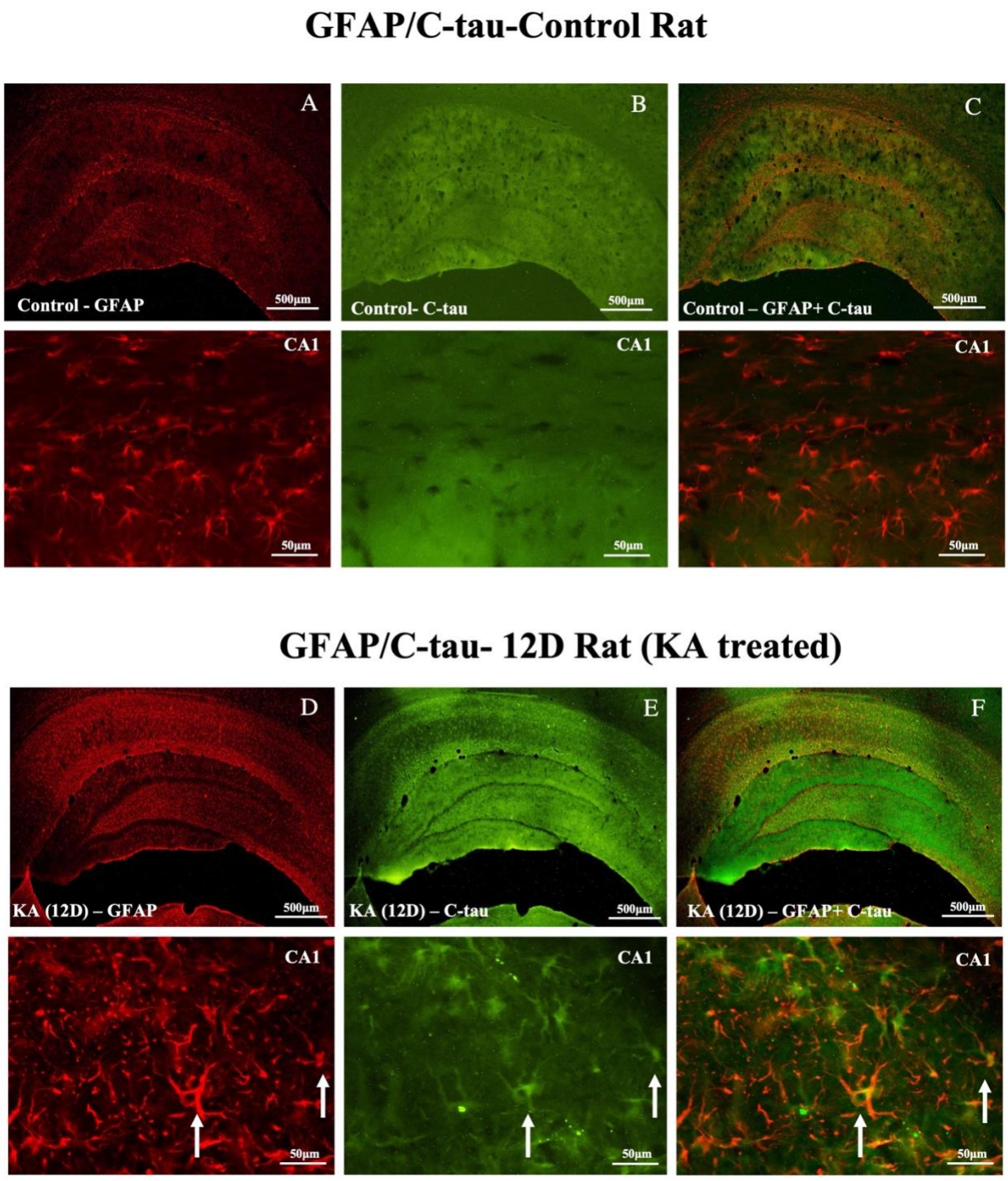

Figure 3.10: Fig (A-F) show the level/expression of astrocytes (GFAP and Cleaved tau) in the hippocampus from control to kainic acid-12D treated rats, with their co-labelled image.

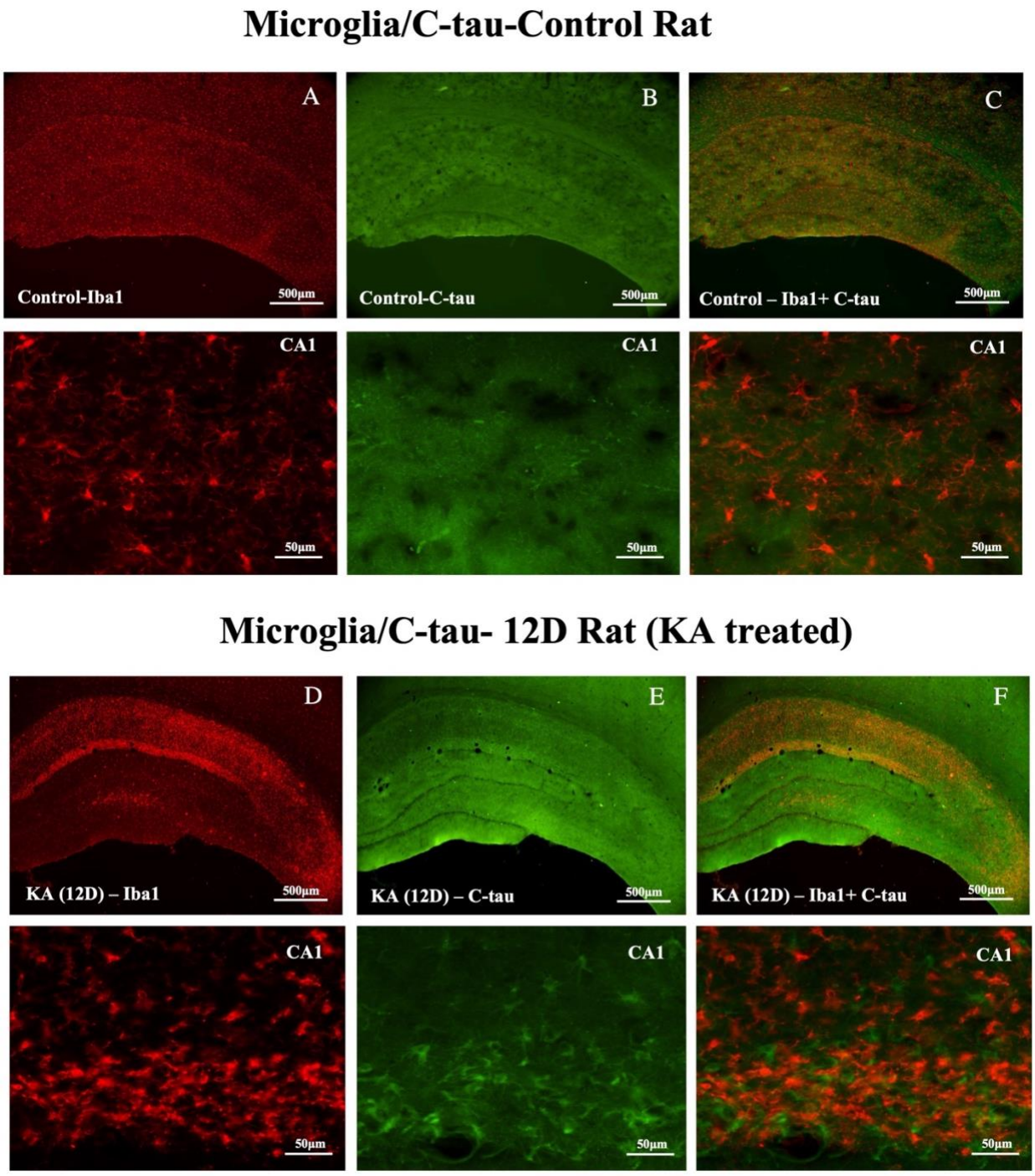

Figure 3.11: Fig (A-F) show the level/expression of microglia (Iba1 and Cleaved tau) in the hippocampus from control to kainic acid-12D treated rats, with their co-labelled image.

4. DISCUSSION

4.1 *Summary*

The present study reveals that various astrocytic markers are differentially altered along with transient increase in the level of total/phospho-tau proteins in kainic acid-induced model of TLE. This is supported by data which show that i) kainic acid treatment time-dependently increase the level and expression of GFAP, vimentin, S100β, Aldh1l1, but not GS, in the hippocampus, ii) GFAP-positive astrocytes co-labelled with vimentin, S100β, Aldh1l1, but not GS, are also found to increase in kainic acid-treated hippocampus, iii) the levels of total and phospho-tau proteins are transiently enhanced in the hippocampus of kainic acid-treated rats and iv) the levels and expression of cleaved-tau which is evident in a subset of GFAP positive astrocytes are increased following kainic acid treatment. These results, taken together, suggest a differential role for various astrocytic subpopulations and tau protein in regulating degeneration of neurons and/or seizure in kainic acid model of TLE. Given the neuropathological parallels between kainic acid administration and human mTLE, it is possible that different subsets of astrocytes may have unique role in disease pathogenesis.

4.2 *Role of astrocytes in kainic acid model of TLE*

Under normal condition, an astrocytic network is present in virtually all regions of the brain in a continuous, non-overlapping manner. Their active role in synaptic transmission has long been recognized in the concept of tripartite synapse where astrocytic processes enveloping neuronal synapses release neuroactive molecules such as glutamate, adenosine, ATP, D-serine and GABA directly influencing synaptic activity (Perea et al., 2009; Bozic et al., 2021). In addition to the active role *via* gliotransmission, astrocytes are known to influence i) the formation and pruning of

the synapses during development and in adult brains (Sofroniew and Vinters, 2010), ii) provide trophic support for neuronal survival and neurite outgrowth by secreting growth factors such as nerve growth factors, brain-derived neurotrophic factors etc. (Kıray et al., 2016), iii) provide support to neurons by synthesizing metabolic substrates such as glycogen, steroids and lipoproteins (Brown and Ransom, 2007; Wang and Bordey, 2008), iv) regulate extracellular concentrations of ions and neurotransmitters (Wang and Bordey, 2008), v) maintain fluid and pH homeostasis at the synaptic cleft (Sofroniew and Vinters, 2010), vi) involved in the formation of blood brain barrier (BBB) and regulation of cerebral blood flow (Abbott et al., 2006; Marina et al., 2020), vii) promote maturation and differentiation of oligodendrocytes precursor cells and assist in the formation of myelin (Salem et al., 2015) and ix) involved in the clearance of waste as a part of the glymphatic system (Jessen et al., 2015). The functional heterogeneity not only suggest the existence of different subtypes of astrocytes that may vary spatiotemporally, morphologically and physiologically between different brain regions (regional diversity) but also within the same region of the brain (termed as local heterogeneity). The most apparent regional differences are evident in the morphology, molecular profile and functionality of the protoplasmic and fibrous astrocytes of the gray and white matters. While cell bodies of protoplasmic astrocytes are extremely ramified allowing them to contact numerous synapses simultaneously, the fibrous astrocytes are relatively small and have fewer branching points in any given region of the brain (Oberheim et al., 2012; Lundgaard et al., 2014; Matias et al., 2019). Furthermore, protoplasmic astrocytes located in different brain regions of the gray matter are also found to be diverse i.e., astrocytes in the hippocampus and cerebellum express high levels of GFAP unlike protoplasmic astrocytes of cerebral cortex and striatum (Hewett, 2009; Bozic et al., 2021). Local heterogeneity such as expression of glutamate transporters, receptors and ion channels may allow astrocytes in each brain

region to carry out different functions. Nonetheless, it is still a matter of debate whether astrocyte diversity is the consequence of different astrocyte lineages and/or expression of different molecules that can be distinguished under physiological and/or pathological conditions (Zhang and Barres, 2010; Miller, 2018). Although GFAP is a classical marker for the identification of astrocytes localized in different brain regions, it is not found to be expressed by all astrocytes either in normal or diseased/injured brains (Hol and Pekny, 2015; Matias et al., 2019). Other astrocytic markers include the vimentin, GS, S100β, Aldh1l1, glutamate transporters (i.e., EAAT1 and EAAT2), nestin, aquaporin 4, Sox9 and connexins 30 and 43 (Sofroniew and Vinters, 2010; Matias et al., 2019; Bozic et al., 2021). These markers *albeit* suggest some kind of selectivity of astrocytic protein expression, it remains unclear if it is intertwined with functional diversity associated with astrocytes. This is simply due to lack of a universal astrocyte marker that enables us to identify intrinsic differences in astrocytes based on their functionality and/or distributional profile in the central nervous system (Matias et al., 2019; Bozic et al., 2021). It is believed that genetic identification of the transcriptional profile of astrocytes paired with a systemic evaluation and comparison of different markers may lead to a better understanding about the role of various subsets of astrocytes in the central nervous system under physiological as well as pathological conditions including TLE.

Consistent with earlier studies (Kar et al., 1997; Ben-Ari and Cossart, 2000; Sarkisian, 2001) we observed that systemic administration of kainic acid can induce degeneration of neurons along with hypertrophy of both microglia and astrocytes in the hippocampus. Degeneration of neurons is evident primarily in CA1-CA3 pyramidal cells and in the hilar region of the dentate gyrus but not much in the granular cell layer (Konishi et al., 1978; Ben-Ari, 1985; Kar et al., 1997; Sarkisian, 2001; Coulter et al., 2002; Vincent and Mulle, 2009). These effects are believed to be mediated by

kainite receptor-induced glutamatergic currents that trigger influx of Ca^{+2} and subsequent activation of downstream signaling cascades including ERK1/2, calpain and caspase (Ben-Ari and Cossart, 2000; Bengzon et al., 2002). The magnitude of cell loss not only depends on the extent and duration of seizure discharges but also on the doses of kainic acid used in the study (Vincent and Mulle, 2009; O'Dell et al., 2012). Accompanying loss of neurons, reactive astrocytes (astrogliosis) are considered to be a hallmark of kainic acid model of TLE which is reflected by cellular hypertrophy and increase expression of GFAP as evident in the present study. Furthermore, reactive astrocytes were found to exhibit alterations in various other markers including GFAP, vimentin, S100β, Aldh1l1, but not GS, in the hippocampus of kainic acid-treated rats. Previous reports indicate that functional and morphological changes observed in reactive astrocytes following seizure may have a dual nature based on the underlying pathological features. The initial response could be a part of glial compensatory mechanism following damage to the nervous system and leading to the reduction in neuronal excitability to restore homeostatic balance during seizures (Vargas-Sánchez et al., 2018; Verhoog et al., 2020; Lu et al., 2021). Depending on the extent of neuronal damage, reactive astrocytes can secrete neurotrophic factors to support injured neurons, phagocytize synapses, clear debris and can help in axon regeneration (Sofroniew, 2005; Becerra-Calixto and Cardona-Gómez, 2017). This protective initial response in the presence of continuing seizure may lead to harmful effects which can alter metabolic activity, ion buffering, neurotransmitter uptake, exacerbate inflammatory response and promote neuronal death as well as maintenance/facilitation of seizure activity (Binder and Steinhäuser, 2006; Vargas-Sánchez et al., 2018). Recent studies have shown that activated microglia are able to induce neurotoxic phenotype in astrocytes by secretion of pro-inflammatory mediators such as IL1α, TNFα and complement component subunit 1q (C1q) (Liddelow and Barres, 2017; Liddelow et al., 2017; Verhoog et al.,

2020). Our study clearly indicates that the levels of complement cascade protein (C3) is increased in a subset of reactive astrocytes, suggesting that a crosstalk between microglia and astrocytes may be involved not only in the degeneration of neurons but also in maintaining seizure activity.

At present, functional significance of astrocytes during seizure and/or degeneration of neurons remain unclear. Given the heterogeneity of astrocytes as evident from our study showing overlapping as well as distinct populations of GFAP with other markers such as vimentin, GS, S100β and Aldh1l1, it is likely that different subtypes of astrocytes may influence different facets of TLE pathogenesis. This is of significance as multiple analyses have revealed that not all astrocytes express GFAP (Hol and Pekny, 2015; Matias et al., 2019). In keeping with an increased levels/expression of GFAP, we observed an enhanced vimentin level in hypertrophic reactive astrocytes from 2day post-treatment onwards in kainic acid-treated hippocampus. Earlier studies have shown that levels/expression of vimentin are increased in hypertrophic reactive astrocytes during inflammation and neurodegeneration in animal models of brain injury (Peković et al., 2005; Voskuhl et al., 2009). There is some evidence that vimentin and GFAP expression is upregulated in both A1 and A2 astrocytes following brain injury (Liddelow et al., 2017; Bozic et al., 2021). Since the increased level of vimentin parallels with GFAP in kainic acid-treated hippocampus, it is possible that vimentin-positive reactive astrocytes may contribute initially to protect neurons which eventually die due to overriding seizure-induced neurotoxic signals. In contrast to vimentin, the number of astrocytes expressing S100β with and without GFAP are increased only at 12 days following treatment with kainic acid. Earlier studies have shown that S100β, a member of calcium binding S-100 protein family, has been shown to play a role in cell-cell interaction, cell cycle regulation, cytoskeleton organization and scavenging toxic substances. There is also evidence that intracellular S100β can stimulate cell proliferation, migration along with inhibition of apoptosis,

while extracellular S100β can regulate inflammatory response (Donato et al., 2009; Bongarzone et al., 2017) suggesting a rather protective role following injury/degeneration. Since S100β expression in activated astrocytes is increased at 12day post-treatment when significant loss of neurons is already evident, it is likely that S100β-positive astrocytes may contribute to the inflammatory response and/or scavenging substances resulting from degeneration of neurons. Accompanying S100β, astrocytes expressing Aldh1l1 with and without GFAP are increased in the hippocampus at 12 days following kainic acid treatment. The significance of enhanced astrocytic Aldh1l1, a folate metabolizing enzyme mediating a variety of reactions including *de novo* nucleotide biosynthesis and influencing growth/regeneration of neurons, is of interest as Aldh1l1 expression is not found to be altered either following brain injury and/or neurodegenerative diseases (Yang et al., 2011; Bozic et al., 2021). Since kainic acid treatment results in the proliferation of mossy fibers involves in the propagation of seizures (Wang et al., 2018) it remains to be determined if enhanced level/expression of Aldh1l1 in astrocytes may have a role in influencing the growth of mossy fibers and/or seizure propagation following kainic acid treatment. Interestingly, the levels/expression of GS, in contrast to GFAP, vimentin, S100β and Aldh1l1, did not alter at any time following kainic acid treatment. Since GS, a glutamate catabolizing enzyme, is involved in glutamate-glutamine cycle, it has been reported to play a role in the degeneration of neurons *via* excitotoxicity. This is supported partly by the expression of GS in astrocytes during injury triggered by inflammation (Rose et al., 2013; Jakovljevic et al., 2019; Bozic et al., 2021). Considering the evidence that glutamate-mediated excitotoxicity plays a critical role in the degeneration of neurons following kainic acid treatment, it is possible that astrocytes expressing GS following kainic acid treatment may regulate extracellular glutamate levels to protect neurons against neurotoxicity. Collectively, the present study revealed that various subtypes of astrocytes

expressing different markers are differentially altered following kainic acid treatment, but their functional significance in relation to propagating seizures and/degeneration of neurons remains to be determined from future studies. Additionally, it is of interest to determine how certain other markers of astrocytes such as EAAT1, EAAT2, nestin, aquaporin 4, Sox9 and connexins 30 and 43 (Matias et al., 2019; Bozic et al., 2021) are altered following kainic acid treatment and their implication, if any, kainic acid-induced animal model of TLE.

In addition to aforementioned markers, we also observed that levels/expression of complement cascade C3 and calcium binding protein S100A10 are differentially altered in the reactive astrocytes in the hippocampus of kainic acid-treated rats. While the level of C3 is increased at 2- and 12-days post-treatment, the level of S100A10 is found to be enhanced at all time points studied (i.e., 12hr to 12days). An increase in C3 expression has recently been reported following intracerebral injection of kainic acid in wild type mice, whereas $C3^{-/-}$ mice showed decrease in degenerating neurons (Wei et al., 2021). There is evidence that astrocytes expressing C3 may exhibit loss of normal functions such as ability to induce synapse formation and gain of new harmful function due to increased expression of many classical component cascade genes and secretion of certain lipoprotein molecules (APOE and APOJ) that are toxic to synapses (Liddelow et al., 2017; Matias et al., 2019; Guttenplan et al., 2021). Conversely, astrocytes expressing S100A10 are believed to secrete neuroprotective cytokines such as thrombospondins (TSPs) suggesting a protective role under certain conditions (Papadopoulos et al., 2004; Manley et al., 2009; Matias et al., 2019). Since reactive astrocytes in animal models of TLE have been suggested to be involved initially in restoring homeostatic balance which in the presence of continues seizure led to harmful effects (Binder and Steinhäuser, 2006; Vargas-Sánchez et al., 2018; Verhoog et al., 2020; Lu et al., 2021), it is possible that astrocyte subtypes expressing S100A10 and C3 may partly

be involved in mediating respective roles in kainic acid-induced animal model of TLE. Although this adds another layer of complexity to the immense possibilities of phenotypes that astrocytes can acquire after an insult, the existence of a gradient between these two different phenotypes (Matias et al., 2019; Bozic et al., 2021; Escartin et al., 2021) may likely represent the intricacy of changes encountered following disease pathology and/or injury to the nervous system.

4.3 *Role of tau protein in kainic acid model of TLE*

Tau is well known to have a significant influence on neuronal activity and seizures. Several studies have shown that tau to be integral to hyperactivity in animal models of TLE (Roberson et al., 2007, 2011; Sánchez et al., 2018). This is supported by the evidence that knock-down of tau is neuroprotective and ameliorates seizure activity, whereas tau overexpression results in hyperexcitability and seizures (García-Cabrero et al., 2013; Holth et al., 2013). Consistent with these results we observed that total (Tau-5 and Tau-1) and phospho-tau (using AT270 and PHF1 antibody) levels are significantly increased at 12hr and the decreased markedly by 12days after treatment with kainic acid. Phosphatase treatment validates the specificity of phospho-tau increase observed following kainic acid treatment. An earlier study reported that transient increase in total tau level observed following kainic acid treatment did not correspond to any significant change in tau mRNA and thus likely not the consequence of an increased transcription (Alves et al., 2019). Several studies involving animal models of TLE have also reported that a transient increase in phospho-tau levels is accompanied by an enhanced levels/activation of tau kinases such as CDK5 and GSK-3β as well as decrease level/activity of phosphatase PP2A (Liang et al., 2009; Gangarossa et al., 2015). There is evidence that administration of kainic acid (30mg/kg. I.P.) leads to an increased phosphorylation of tau protein at several sites (Thr^{231}, Ser^{199} and Ser^{396}) after 3days post-treatment in male CD10mice. This is accompanied by transient increase in the

levels/activation of GSK3β and CDK5 which are higher at 3days post treatment and then gradually decrease by 7days (Crespo-Biel et al., 2007). Tau phosphorylation and aggregation has also been observed following intra-amygdala kainic acid-induced seizure in male C57BL6 mice (Alves et al., 2019). Pilocarpine-induced chronic TLE in 3xTg-AD mouse model has been shown not only to increase the formation of neuritic plaques but also enhanced intraneuronal aggregation of phosphorylated tau in the temporal cortex compared to normal control mice (Yan et al., 2012). There is also evidence that reduction or ablation of tau can protect mice against seizures induced by kainic acid as well as other pharmacological agents/drugs (Roberson et al., 2007; Ittner et al., 2010; Holth et al., 2013). Findings from animal studies have shed lights on the contribution of tau protein in human mTLE (Sen et al., 2007; Thom et al., 2011; Dyment et al., 2015; Tai et al., 2016; Liu et al., 2017). Hyperphosphorylated tau protein identified by immunohistochemistry using AT8 staining has not only been upregulated but also been detected in the form of neuropil threads, NFTs and pre-tangles in the temporal lobe of TLE patients who underwent anterior temporal lobe resection (Tai et al., 2016). This is accompanied by an increased expression of CDK5 and GSK-3β indicating a potential role for these kinases in phosphorylation of tau protein in TLE patients (Liu et al., 2017). Although the functional significance of hyperphosphorylated tau in TLE remains to be defined, it is likely to contribute to a series of events such as neuronal network reorganization including mossy finer sprouting (Tian et al., 2010; Huang et al., 2013), aberrant neuronal cell migration with hippocampal granule cell layer dispersion (Kandratavicius et al., 2013), glutamate receptor (NR2B)-mediated glutamate release (Decker et al., 2016) and neuroplasticity changes (Sotiropoulos et al., 2017) that are implicated in the development of TLE pathogenesis.

In addition to phosphorylation, tau protein undergoes a variety of other post-transcriptional modifications including truncation. The enzymes calpain and caspase are primarily responsible for

the cleavage of tau leading to formation of cleaved tau that are believed to be toxic and contribute to formation of NFTs (De Calignon et al., 2010; Hanger and Wray, 2010). Caspase cleaves tau at asp421 (D421) in the C-terminus domain, generating two peptides: a short COOH-terminal peptide with undefined functions and a truncated tau protein (Δ421) that is considered to play a role in a variety of neurodegenerative diseases (Hanger and Wray, 2010; Biundo et al., 2017). Kainic acid administration activates caspase 3 which plays a role in the degeneration of neurons as the inhibitor of the enzyme prevents neuronal damage (Faherty et al., 1999; Henshall et al., 2000; Kondratyev and Gale, 2000). Our study revealed that steady-state levels of caspase-cleaved tau is significantly increased and is evident primarily in GFAP-labelled astrocytes, but not in microglia, in the hippocampus of kainic acid-treated rats. This is consistent with earlier results which showed an enhanced levels of cleaved-tau following administration of kainic acid as well as in traumatic brain injury (TBI) and methamphetamine (METH) induced neurotoxicity (Wallace et al., 2003; Gabbita et al., 2005). Furthermore, toxicity induced by trimethyltin and amphetamine showed the presence of cleaved-tau in GFAP-labelled astrocytes as observed in the present study (Straiko et al., 2006, 2007). At present, however, it is not clear if the expression of caspase-cleaved tau is associated with any specific subsets of astrocytes and its significance, if any, in TLE pathogenesis. Earlier studies have shown that caspase-mediated cleavage of tau protein might be involved in the etiology of AD (Gamblin et al., 2003; Rissman et al., 2004), chronic traumatic encephalopathy (Kanaan et al., 2016), TBI (Glushakova et al., 2018) but their functional significance on context of degeneration of neurons and/or development of disease pathology remains to be established from future study.

Like most studies, the current study has its own limitations. The use of vimentin as a marker for astrocytes has some constraint as it is known to be expressed by other cell types such as epithelial

cells and fibroblast (Castro-Muñozledo et al., 2017). Furthermore, S100β is also expressed by a subset of oligodendrocytes (Du J et al., 2021). We could have co-stained vimentin or S100β with other cell specific markers to find out the extent of vimentin and S100β co-localization in our study. This will certainly be addressed in our future work. The results obtained in the present study, taken together, indicate differential role for various subsets of astrocytes as well as tau protein in degeneration of neurons and/or seizure in kainic acid model of TLE. Given the pathological similarities between kainic acid animal model of TLE and human mTLE, it is likely that astrocytes and tau protein may have important roles in the development of TLE pathogenesis.

4.4 *Future direction*

1) The use of single cell RNA sequencing (scRNAseq) will definitely aid in the identification of a more complete image of the astrocyte profile in the hippocampus of kainic acid's TLE animal model. Thus a better understanding of astrocyte subpopulations may help in the identification of regional activation patterns in the future (Anderson et al., 2014). Moreover, using a more advanced co-culture model (Qiu et al., 2018), it may be possible to identify the effects of distinct cell types in TLE pathogenesis.

2) We can examine if increased levels of total tau are associated with an increased mRNA levels in the treated *vs* untreated kainic acid hippocampus. Similarly, the role of different tau kinases and phosphatases in the hippocampus might be an important aspect to study.

3) The significance of cleaved tau in astrocytes in kainic acid-mediated toxicity can be studied using astrocytes as well as mixed astrocyte/neuronal cultures.

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