The role of β-amyloid peptides in kainic acid-induced toxicity and its implications in Mesial Temporal Lobe Epilepsy

by

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ABSTRACT

Kainic acid is a non-degradable analogue of the excitatory neurotransmitter glutamate that, when injected systemically into adult rats, can trigger seizures and progressive neuronal loss in a manner that mirrors the neuropathology of human mesial temporal lobe epilepsy (MTLE), most prevalent form of partial epilepsy. However, the biomolecular mechanisms responsible for the neuronal loss that occurs as a consequence of this treatment remain elusive. Recent studies from our lab have shown that kainic acid administration can lead to increased levels/processing of amyloid precursor protein (APP) in activated astrocytes leading to enhanced production of amyloid-β (Aβ) peptides, which are known to play a critical role in the neurodegeneration observed in Alzheimer's disease. At present, however, the functional consequences of Aβ peptides on kainic acid-induced loss of neurons remain unclear. Thus, in this study, we seek to establish the potential role of kainic acid-induced astrocytic Aβ peptides on the degeneration of neurons. Our results show that kainic acid treatment of human U373 astrocytoma and rat primary astrocyte cells yields increased levels/processing of APP, resulting in enhanced Aβ production/secretion without compromising cell viability. Additionally, we reveal that kainic acid induces neuronal loss more in neuronal/astrocyte co-cultures than pure neuronal cultures, and this is attenuated by precluding Aβ production. Furthermore, using selective ionotropic glutamate receptor antagonists, we show that the kainate receptor is specifically responsible for facilitating enhanced amyloidogenesis in astrocytes, thus implying an important role for this underexplored receptor in a disease context. These results suggest that Aβ peptides derived from astrocytes may have a role in kainic acid-induced neurodegeneration. Since administering kainic acid can recapitulate the main pathological features of MTLE, it is possible that the mechanisms similar to those observed in this study may also be responsible for the degeneration of neurons in this disease.

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ABBREVIATIONS

Aβ	Amyloid-β peptide
ACET	(S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5- methylpyrimidine-2,4-dione
AD	Alzheimer's disease
ADAM10	A Disintegrin and Metalloprotease 10
AMPA	alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropionic acid
ANOVA	Analysis of varience
APH1	Anterior pharynx-defective phenotype 1
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
ATG5	Autophagy protein 5
BACE1	β-site APP cleaving enzyme1
BSA	Bovine serum albumen
CHAPSO	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CNS	Central nervous system
α/βCTF	C-terminal fragment-alpha/beta
D-AP5	D-2-amino-5-phosphopentanoic acid
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DMEM	Dulbecco's modified Eagles medium
EAAT-1/2	Excitatory amino acid transporter-1/2
ECL	Enhanced chemiluminescence
EDTA	Ethelynediaminetetracetic acid
ELISA	Enzyme-linked immunosorbence assay
ER	Endoplasmic reticulum
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GYKI 52466	1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride
IBO	Ibotenic acid

IDE	Insulin degrading enzyme
KA	Kainic acid
LC3	Microtubule-associated protein 1A/1B light-chain 3
MTLE	Mesial temporal lobe epilepsy
MTT	3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide
NEP	Neprilysin
NFT	Neurofibrillary tangles
NMDA	N-methyl D-aspartate
PBS	Phosphate-buffered saline
PEN2	Presenilin enhancer 2
PS1/PS2	Presenilin 1/Presenilin 2
RIPA	Radioimmunoprecippitation assay buffer
ROS	Reactive oxygen species
sAPPα/β	Soluble amyloid precursor protein alpha/beta
SDS	Sodium dodecyl sulfate

LIST OF PUBLICATIONS BY THE CANDIDATE

Ourdev D, Foroutanpay BV, Wang Y, Kar S. 2015. The effect of Aβ1-42 oligomers on APP processing and Aβ1-40 generation in cultured U-373 astrocytes. *Neurodegener Dis*, 15:361-8.

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1. Introduction

1.1. ***Epilepsy and seizures overview*****:** Epilepsy is one of the oldest known human conditions and the third most commonly diagnosed medical disorder, affecting approximately 50 million people worldwide (Hirtz et al., 2007). This disease is characterized as the state of having recurring episodes of aberrant neural activity known as seizures, wherein groups of neurons within select brain regions discharge inappropriately in synchronized, repetitive bursts (Hirtz et al., 2007). Seizures arise as a result of a combination of factors, such as an external insult like trauma, hypoxia, stroke, etc., which ultimately lead to neural circuit reorganization that favors neuronal hyperexcitability and reduces inhibition (Scharfman et. al., 2007). This process can subsequently incite the affected neurons to become progressively more vulnerable and seizure-prone, which can further increase the risk of epilepsy. It should be noted that, despite the current understanding of seizure mechanisms, the initial triggering factor is oftentimes unknown in most epilepsy cases; likewise, epilepsy as a condition specifically refers to seizures which appear to be spontaneous – it does not include febrile seizures (due to high body temperature) or those caused as a result of a specific chemical or condition, such as drug withdrawal.

Seizures are typically classified as either partial, which are localized to a particular brain region or area, or general, which propagate throughout and affect the entire brain. Partial seizures are further categorized as either complex or simple, based on whether they are accompanied by alterations in consciousness or not, respectively. Depending on the brain regions involved, seizures can lead to a wide variety of neurological and psychiatric symptoms, such as amnesia, hallucinations, cognitive impairment, mood disorders, and psychosis (Algreeshah et al., 2014; Bancaud et al., 1994). On the other hand, generalized seizures affect the whole brain, and therefore typically manifest as a loss of consciousness coupled with a simultaneous loss of motor control which ranges from brief lapses in muscle tone (atonus) to involuntary twitching (myoclonus) to violent convulsions (tonic-clonic seizures). Notably, different types of seizures can occur concurrently, and partial seizures can potentially propagate into generalized seizures. As a direct consequence, the brain areas affected by seizures may experience compensatory synaptic remodeling, astrogliosis, and neuronal loss (Weiser et al., 2004). Over time, this can lead to chronic neurological damage and cognitive impairment which greatly diminishes one's quality of life.

Likewise, the duration and intensity of a seizure correlates with the severity of potential danger it poses to an individual; the longer the seizure, the greater the risk for lasting neural damage (Oyegbile et al., 2004).

1.2. ***Mesial temporal lobe epilepsy*:** Of the many types of epilepsies, the most common type is mesial temporal lobe epilepsy (MTLE), which accounts for approximately 48% of all epilepsies (Téllez-Zenteno and Hernández-Ronquillo, 2012), and occurs at a rate as frequent as 4-10 cases per 1000 people in developed countries, and 14-57 cases per 1000 in developing and tropical countries. The peak incidence for MTLE occurs in early childhood and adolescence, tapering off around middle age. Interestingly, the rate of occurrence for MTLE drastically increases again after the age of 60 and is especially prevalent as a co-morbidity in populations with dementia (Chin and Scharfman, 2013). This often leads to issues with proper diagnosis, as early cognitive impairment in dementia can oftentimes be confused with the types of psychiatric symptoms that result from MTLE (Noebels, 2011). Hence, it is possible that the reported frequency of epilepsy is significantly underestimated and that the true incidence of MTLE is much higher in demented and elderly populations.

Unfortunately, MTLE is also the most resistant to pharmacotherapy. Whereas other types of epilepsies can typically be controlled by anti-epileptic drugs, such as acetazolamide and levetiracetam, most MTLE cases either become refractory or don't respond well to treatment to begin with – in these situations, the best recourse for MTLE is often surgery (Al Otabi, et al., 2012). Surgery, however, is not without its drawbacks, in that there are inherent risks for the patient associated with invasive neurosurgical procedures, which can sometimes lead to behavioral changes and memory loss as unintended consequences (Acosta et al., 2008; McLachlan et al., 1992). Given the increasing prevalence of epilepsy with old age and the lack of an effective, long-term treatment, the projected increases in the global average age demographics renders this condition one of the major medical challenges of our time (Acharya and Acharya, 2014; Houser et al., 1996). As such, understanding its molecular mechanisms and etiology is critical to opening up new avenues to therapeutic approaches.

1.3. ***MTLE pathophysiology*:** MTLE is so named because the seizures involved in this condition originate from the mesial temporal lobe structures of the brain; specifically, the hippocampus, surrounding cortical areas and, less frequently, the amygdala (Weiser et al., 2004). Because these structures are most intimately associated with memory formation, spatial perception, and emotional processing, MTLE most often involves complex partial seizures which generate syndromes such as fixed staring, impaired consciousness and memory, and altered sensations such as *déjà vu* (Kaplan et al., 2005; Bancaud et al., 1994). In terms of pathophysiology, MTLE is most commonly associated with hippocampal sclerosis (Weiser et al., 2004) (Figure 1.1). This condition results from progressive adverse changes in the hippocampal formation as a result of epileptic activity, including but not limited to:

- widespread neuronal loss and gliosis (which occurs throughout the hippocampus to varying degrees, but is particularly prominent in the CA1 and CA3 regions)
- synaptic reorganization, especially involving mossy fibers and supragranular layer of the dentate gyrus formation
- functional and structural changes in astrocytes and microglia
- granule cell dispersion; and
- atrophic induration (a shrinkage and hardening) of the hippocampus

Control over excitability in different areas of the brain is mediated in part by the precise arrangement of neurons within a given neural circuit (Hazra et al., 2013). In the hippocampus, for instance, glutamatergic projection neurons are connected to elaborate circuits involving inhibitory interneurons, thus forming multiple levels of feed-forward and feedback inhibition that modulates their overall activity; changing the circuitry can trigger changes in the overall activity involved. Accordingly, the pathological changes triggered by hippocampal sclerosis can directly influence network excitability by either stimulating the formation of excitatory circuits through synaptic reorganization and axonal sprouting, or decreasing the presence of inhibitory circuits through cell death (Palop et al., 2006). The overall consequence is that the sclerotic hippocampus becomes progressively more epileptogenic over time. This is supported by depth electrode recordings from patients with MTLE which indicate that most seizures specifically originate from sclerotic hippocampal tissue; conversely, surgical removal of these areas terminates the occurrence of seizures in 85% of patients (Al-Otaibi et al., 2012).

1.4. *Glutamate and molecular mechanisms of excitotoxicity*: On a molecular level, these pathological changes are mediated, at least in part, by the neurotransmitter glutamate. Glutamate is the principal excitatory neurotransmitter in the mammalian nervous system and an essential component of neural networks in the hippocampus responsible for memory formation and learning. Its function is predominantly facilitated by three ionotropic receptors named after their respective selective agonists – N-methyl D-aspartate (NMDA), alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropionic acid (AMPA), and kainic acid (KA) receptors; glutamate function is further modulated by the activity of eight metabotropic glutamate receptors (mGluRs), as well as other neurotransmitter molecules such as the inhibitory γ-aminobutyric acid (GABA) (Kew and Kemp, 2005). The activation of ionotropic receptors by glutamate triggers an influx of sodium cations which drive action potentials; furthermore, prolonged glutamate stimulation can result in the influx of Ca^{2+} either specifically through the NMDA receptor itself, or indirectly via the opening of other calcium channels. Intracellular Ca^{2+} subsequently acts as a secondary messenger molecule for the propagation of intracellular signaling cascades that modify the neuron's response to stimuli. Under physiological conditions, this forms a mechanism for activity-dependent changes in gene expression and protein synthesis which alter the connectivity and synapses between stimulated neurons. However, it also presents a biological caveat – if the intracellular Ca^{2+} ion concentration rises above a certain threshold for an extended period of time, the neuron may instead be forced to undergo cell death through either necrotic or apoptotic mechanisms – a process termed excitotoxicity (Olney et al., 1986). Large influxes of Ca^{2+} can inappropriately activate a myriad of calcium-dependent enzymes, including proteases and phosphatases which can degenerate intracellular compartments (Berliocci et al., 2005). Influxes of Ca^{2+} typically trigger a rapid uptake by the mitochondria, which act as sinks to buffer excessive intracellular concentrations. However, a prolonged flood of Ca^{2+} can overload and damage the mitochondria, causing a disruption in membrane potential, loss of ATP production, and an increase in membrane permeability. A direct consequence of permeability is the leakage of cytochrome c into the cytoplasm, leading to the formation of the apoptosome complex and the subsequent initiation of the caspase-dependent apoptotic cell death. Alternatively, the loss of ATP production in the mitochondria also coincides with a rapid rise in reactive oxygen species (ROS), which themselves act as agents that induce intracellular damage and push a cell towards necrotic cell death (Dong et al., 2009; Choi, 1992). Collectively, these events underlie the mechanism by which excessive neural activity can precipitate neurodegeneration and epileptogenesis. In MTLE, for instance, baseline extracellular hippocampal glutamate levels are five times higher than in non-MTLE patients (Cavus et. al., 2005); notably, these levels increase 30-fold during a seizure and remain markedly elevated for approximately 20 minutes after (During and Spencer, 1993). The role of this mechanism in MTLE is further supported by evidence that administration of glutamate analogues, such as KA, can induce seizures and neuronal loss in animal models in a manner that mimics the pathology observed in MTLE patients (Olney et. al.1986; L'evesque, M. and Avoli, 2013). Conversely, glutamate receptor antagonists such as ketamine are capable of reducing seizure-induced neuronal loss in animal models of MTLE (Eid et al., 2008; Seifert et al., 2010). Collectively, these findings highlight the importance of aberrant glutamate neurotransmission as a critical initiation point in the excitotoxic cascade (Sattler and Tymianski, 2001) and a key element in the pathological development of epilepsy.

1.5. ***Glutamate regulation and the importance of astrocytes*****:** Under normal conditions, the presence of glutamate in the synapse is tightly controlled by reuptake mechanisms (Anderson and Swanson, 2000). The bulk of this reuptake is mediated by astrocyte cells via the sodium-coupled glutamate transporters, excitatory amino acid transporter-1 (EAAT-1) and EAAT-2. Once inside the astrocyte, glutamate is rapidly converted to glutamine by the enzyme glutamine synthase and transported back to the neurons, where it is re-converted to glutamate and packaged into pre-synaptic vesicles. As such, glutamate in the central nervous system (CNS) is constantly recycled and its homeostasis is maintained by astrocytes. Astrocytes are therefore integral to proper neural communication; dysfunctions in either the transport function of EAAT-2, or the glutamine turnover by glutamine synthase has been identified as a key aspect of various neurodegenerative disorders, including epilepsy (Tian et al. 2005). The expression of both EAAT-2 and glutamine synthase have been shown to be reduced in sclerotic hippocampi (Proper et al. 2002, Eid et al. 2004), and the inhibition of the EAAT-2 homolog glutamate transporter 1 (Glt-1) reduces the threshold for epileptiform activity (Campbell and Hablitz, 2004), whilst its knock-out induces spontaneous seizures and MTLE-like pathology in mouse models (Tanaka et al., 1997). Astrocytes are also shown to release glutamate in a Ca^{2+} dependent manner (Tian et al., 2005), which highlights a possible feed-forward mechanism towards excitotoxicity in an epileptic environment.

Furthermore, astrocytes may become inflamed or "activated" in response to CNS injury or pathological stress. In this state, astrocytes become hypertrophic, develop processes, and begin to express a number of inflammatory markers such as Interleukin-1β (IL-1β) and Glial Fibrillary Acidic Protein (GFAP) (Johnstone et al., 1999), whilst simultaneously modifying the majority of their metabolic functions, including maintaining glutamate homeostasis. In sclerotic hippocampi, neuronal loss is accompanied by an 80% increase in glial density, many of which express the reactive marker GFAP (Blumke et al., 2002). Collectively, given the vital role astrocytes play with regards to glutamate homeostasis, it is likely that these reactive cells are critical in propagating epileptiform activity by exacerbating aberrant glutamatergic neurotransmission.

1.6. *MTLE and amyloid-beta peptides*: Despite an understanding of the general epileptogenic processes by which MTLE can develop, many aspects of MTLE pathology have yet to be fully elucidated and described. Moreover, the factor which initiates MTLE development is oftentimes unknown. Since many different neural insults, such as stroke or traumatic brain injury, can potentially lead to mesial temporal seizures, it is possible that these different conditions can potentially share overlapping mechanisms of pathology with MTLE.

Recently, given its increased incidence in aged populations, there has been a developing interest in the relationship between MTLE and Alzheimer's disease (AD). AD is an insidious neurodegenerative condition which begins as mild cognitive impairment and lapses in memory, and progresses to the eventual loss of all higher cognitive functions (O'Brien & Wong, 2011; Clippingdale et al., 2001). AD is also the leading cause of dementia, affecting approximately 44 million worldwide, and its incidence increases exponentially after the age of 65. One of the hallmark characteristics of AD is the development of extracellular deposits in the brain parenchyma that consist predominantly of the protein amyloid-β (Aβ); these deposits build up initially in the entorhinal cortex and hippocampus of affected patients, and subsequently spread to higher cortical areas (Wragg and Jeste, 1989; Braak and Braak, 1991). Remarkably, AD also frequently features temporal complex partial seizures as a co-morbidity (Powell 2014). These seizures are remarkably similar to the ones present most commonly in MTLE and generate similar cognitive symptoms; so much so, that differentiating between the early stages of AD and MTLE is sometimes difficult (Noebells, 2011; Scharfman, 2012a).

Although seizures and dementia are traditionally considered distinct disorders, there are several lines of evidence which suggest that there are shared mechanisms of pathology between the two, and that furthermore these mechanisms involve the Aβ protein. These include the observation that patients with AD exhibit seizures at frequencies that are far greater than age-matched reference populations (Chin & Scharfman, 2013). This is especially true for patients with familial early-onset form of the disease, which is explicitly caused by mutations that lead to the overproduction of Aβ (Scarmeas et al. 2009). Likewise, patients with Trisomy 21 (Down's syndrome) are also known to be highly susceptible to MTLE; because of their extra copy of chromosome 21, these patients have an increased neural load of Aβ and ubiquitously develop AD-like symptoms by the age of 45. These observations have been further confirmed by studies which indicate transgenic mice and rats overexpressing Aβ peptides may develop spontaneous non-convulsive seizures (Mohajeri et al., 2002; Del Vecchio et al., 2004; Palop et al., 2007; Westmark et al., 2008; Lalonde et al., 2012). Furthermore, biopsies from patients with MTLE indicate that some epileptic brains are subject to developing amyloid deposits similar to the neuritic plaques that are characteristic of AD (Mackenzie and Miller, 1994). Soluble Aβ can modulate glutamatergic signaling, thereby rendering hippocampal neurons more susceptible to excitotoxicity and thus facilitating the occurrence of epilepsy (Palop and Mucke, 2010). Likewise, prolonged activation of the NMDA receptor is known to enhance amyloidogenic processing of APP, leading to the production and secretion of more Aβ (Lesne et al., 2005; Bordji et al., 2010; Noebels, 2011; Rush and Buisson, 2014). Whether the causative factor in each case is the seizures or the Aβ remains controversial. It has been shown that Aβ triggers glutamate release in the hippocampus and exacerbates neuronal susceptibility to KA-induced neurotoxicity (Kabogo et al., 2010; Revett et al., 2013); conversely, it is possible that seizures themselves can trigger Aβ pathology, as studies from our lab as well as others have found that KA treatment increases the expression of APP and several of its processing enzymes (Siman et al., 1989; Morimoto and Oda, 2003; Kodam et al., 2017). Additionally, blockage of the NMDA receptor via antagonists such as memantine has been found to protect neurons from Aβ toxicity (Song et al., 2008); similarly, it has recently been shown that treatment with the antiepileptic drug levetiracetam ablates most behavioral and cognitive deficits in a rat model of AD based on Aβ overexpression (Sanchez et al. 2012; Xiao, 2016). Despite these findings, much about the relationship between Aβ and MTLE remains unclear, especially with regards to Aβ and neuronal loss in the context of epilepsy. Hence, it is our interest to elucidate this connection, as it may lead to new avenues of medical research and therapeutic development. In particular, we are interested in Aβ metabolism in the context of MTLE, and how this Aβ can affect neurodegeneration.

1.7. ***Amyloid precursor protein metabolism and Aβ biosynthesis*****:** Aβ peptides are generated from proteolytic processing of Amyloid Precursor Protein (APP), an integral membrane protein purported to regulate various cellular processes including cell survival/death, synaptogenesis, synaptic plasticity, neuronal excitability, calcium and metal homeostasis, and cell adhesion (O'Brien and Wong, 2011). APP is constitutively expressed by the brain and can be processed by either one of two pathways (Figure 1.2). The first pathway involves the α-secretase enzyme, a disintegrin and metalloprotease (ADAM 10 or ADAM 17), which cleaves APP within the Aβ sequence to yield a soluble APP fragment (sAPPα) and a membrane-bound C-terminal fragment (CTFα). Since the cleavage occurs within the Aβ sequence, this α-secretase pathway precludes Aβ generation, and is hence non-amyloidogenic, eventually producing a soluble fragment known as P3, which is not known to self-aggregate into oligomers. Alternatively, APP can be cleaved by the β-secretase, β-site APP cleaving enzyme 1 (BACE1), resulting in a C-terminal fragment (CTFβ) which contains an intact Aβ sequence. In both pathways, the corresponding CTFs are subsequently cleaved by the multimeric protein complex γ-secretase, consisting of the aspartyl protease presenilin 1 or 2 (PS1/2) stabilized by three co-factors, nicastrin, presenilin enhancer homolog 2 (PEN2) and anterior pharynx defective 1 (APH1) (Cole and Vassar, 2008; Wolfe, 2010). The enzyme γ-secretase cuts at the C-terminal end of the amyloid sequence; in the case of the amyloidogenic β-secretase pathway, this results in a full-length Aβ peptide. Given the variability of γ-secretase cleavage, the amyloidogenic pathway can generate Aβ peptides between 39-43 a.a. long, with the most common isoforms being $A\beta_{1-40}$ and $A\beta_{1-42}$ (Chavez-Gutierrez et al., 2012). Aβ peptides are synthesized along the endocytic pathway, with the majority of synthesis occurring on endosomes, although some are also generated in plasma membrane, endoplasmic reticulum (ER) and Golgi apparatus. Once produced, Aβ is secreted into the extracellular space, where it aggregates into soluble oligomers, which convert into insoluble fibrils, eventually forming plaques. Of the two most common isoforms of Aβ, $A\beta_{1-42}$ is highly fibrillogenic and more toxic to cells (Selkoe, 2001). However, it is also less common, with $A\beta_{1-40}$ making up about 90% of the all Aβ peptides.

1.8. ***Aβ clearance and degradation*****:** The net amount of Aβ in a cell is not simply a function of APP proteolysis and Aβ production, but is a balance between the relative rates of synthesis and its clearance/degradation. Discussing Aβ elimination is arguably more difficult and complex than its synthesis, predominantly because while only two enzymes are involved in its production, there are many different mechanisms by which Aβ can be degraded or eliminated (Baranello et al., 2015; Saido and Leissring, 2012). These mechanisms include proteolytic degradation, cell-mediated clearance, passive and active transport across membranes. Given the propensity of Aβ peptides to form oligomers and fibrils, the overall rate of aggregation also counts as a sink for monomeric Aβ. Enzyme-mediated proteolysis is arguably the most important form of Aβ elimination; this process is mediated by a diverse array of peptidases which may have overlapping functions and can collectively or individually contribute in a significant manner to the rate of clearance. Two important enzymes particularly worth discussing are Nephrilysin (NEP) and Insulin Degrading Enzyme (IDE). Of the various enzymes that degrade Aβ, NEP is the most extensively characterized (Marr et al., 2004). NEP is a transmembrane zinc metallopeptidase of the M13 family; it is almost exclusively expressed in neurons (as opposed to glia) and typically localizes on presynaptic terminals. NEP alone can have a tremendous effect on cellular Aβ levels, as NEP-knock outs result in a doubling in the load of both $Aβ_{1-40}$ and $Aβ_{1-42}$ (Huang et al., 2006). Its effect appears to be dependent on gene dose, as both genetic and pharmacologically-induced overexpression of NEP can effectively abolish Aβ deposition (Poirier et al., 2006; Leissring et al., 2003). NEP activity is not limited to monomeric Aβ; it can also degrade oligomeric forms of the peptides. This process has been shown to ameliorate some of the cognitive deficits in APP-Tg mice, although such findings have been controversial (Meilandt et al., 2009). Similar to NEP, IDE is a zinc metallopeptidase. However, an important difference lies in its inability to fit oligomeric Aβ into its catalytic site; hence, IDE exclusively degrades Aβ monomers (Chesneau et al., 2000). Another key difference is its expression pattern; IDE is found primarily in the cytosol, as well as various intracellular compartments including endosomes/lysosomes, the ER, and peroxisomes (Caccamo et al., 2005). This enzyme can also be secreted into the brain parenchyma, and hence has potential roles in degrading extracellular Aβ. Other enzymes and protein complexes, including cathepsins B and D, proteasomes, etc. can also contribute to the overall clearance rate of Aβ.

Recently, there has also been increased interest in the role of autophagy in APP turnover and Aβ generation (Zare-shahabadi et al., 2015). Autophagy is a process by which cells degrade damaged or extracellular components, abnormal proteins, and bulk cytoplasmic material. This process involves the envelopment of the particle in question within a double membrane structure, essentially forming a specialized vacuole known as the autophagosome. Once the autophagosome is formed, degradation is induced by fusing the autophagosome with the endosomal/lysosomal system. Key regulators of this process, which are also used as biomarkers for identifying and studying autophagy include the proteins Autophagy Protein 5 (ATG5), Microtubule-associated protein 1A/1B light-chain 3 (LC3), Beclin-1, and p62 (Glick et al., 2010). The autophagic pathway is constitutively active and essential for cellular survival; in neurons, it plays an especially prominent role during states of nutrient deprivation by serving as a means to derive energy by recycling superfluous and non-essential cellular contents. On the other hand, dysfunctions in autophagy have been linked to various diseases; normally autophagosomes are scarcely found in healthy neurons and accumulate in abundance in various neurodegenerative conditions, including AD. Autophagy is an active pathway for APP turnover and Aβ clearance (Nixon 2007). Under conditions of nutrient starvation, γ-secretase components translocate from endosomal/ER compartments to autophagosomes (Tung et al., 2012). This results in the increasing levels of immunoreactive Aβ, its precursor βCTF, and γ-secretase activity. Autophagosomes subsequently fuse with lysosomes to facilitate degradation of their luminal components. Alternatively, it has been suggested that a small pool of autophagosomal Aβ is also excreted into the extracellular space via the dynamic interaction with late-endosomes and exosomes. On the other hand, pathological conditions can trigger Aβ-enriched autophagosomes to accumulate intracellularly.

Overall, the total Aβ load within a cell is governed by various dynamic processes. Likewise, the half-life of APP, its proteolytic intermediates, and its Aβ product varies depending on the cell's state and can be disrupted or manipulated by external factors. Thus far, however, the extent of changes in cellular Aβ metabolism that occur as a result of MTLE has not been evaluated.

1.9. ***Astrocytic involvement in Aβ production and APP metabolism*****:** At present, the bulk of scientific literature focuses on APP metabolism and Aβ generation within the context of neurons. However, astrocytes are no less crucial to this process, as these cells also express an abundance of APP and are capable of generating Aβ (Lesne et al., 2013). Because of their close proximity and association with amyloid plaques in AD, astrocytes were presumed to play a role in mediating the clearance of extracellular Aβ deposits via phagocytosis. This process was demonstrated *in vitro* (Wyss-Coray et al., 2003). However, subsequent studies demonstrated that amyloid clearance is only facilitated by healthy astrocytes, and that astrocytes derived from APP-Tg mouse brains had no such function (Zhao et al., 2011). Instead, reactive astrocytes in AD are shown to express BACE1, acquiring amyloidogenic capabilities. Since astrocytes vastly outnumber neurons, it is possible that they represent a significant, if unappreciated, source of Aβ under pathological conditions. Furthermore, studies by our lab using animal models of MTLE have determined that epileptic activity in the hippocampus triggers the extensive proliferation of GFAP-positive astrocytes which overexpress APP; this process occurs simultaneously with the rapid loss of APP-positive neuronal populations, thereby suggesting that reactive astrocytes potentially supplant degenerating neurons as the primary source of pathological APP processing under epileptogenic conditions (Kodam et al., 2017). However, what involvement astrocytes have with respect to Aβ and MTLE pathology, and whether astrocyte-derived Aβ can facilitate neurodegeneration has hitherto been unexplored.

1.10. *The kainic acid model of MTLE*: Our lab utilizes the KA model of MTLE (Wang et al., 2005; Levesque and Avoli, 2013). KA is a non-degradable analog of glutamate, and a potent excitotoxin and epileptogen. This chemical exerts its effects by specifically activating kainate receptors, leading to subsequent influxes in Ca^{2+}, ROS production, mitochondrial dysfunction and other aforementioned excitotoxic processes culminating in neuronal necrosis and apoptosis (Ben-Ari and Cossart, 2000). In animal models, systemic administration of KA results in the selective degeneration of hippocampal neurons, yielding a well-characterized seizure syndrome that closely mirrors the effects of MTLE in humans. Kainate receptors are most abundant in the CA3 region of the hippocampus, and because of the connectivity in this region, KA administration results in extensive damage within the CA1, hilus, and CA3 areas (Vincent and Mulle, 2009). KA-induced neuronal death is further accompanied by extensive astrogliosis in this region. This gliosis is indicated as the steady increase in GFAP-positive cells beginning as early as 24h following administration (Kodam et al., 2017).

The KA model of MTLE has some inherent advantages over other models of epilepsy such as NMDA and ibotenic acid (IBO). Both NMDA and IBO act in a non-specific manner on many different cell types, and oftentimes fail to result in pervasive seizures. Likewise, KA confers a degree of flexibility over models such as kindling, since KA can be applied in both *in vivo* and *in vitro* experimental paradigms, generating a consistent, characteristic set of symptoms which can be assessed over an extended period of time. KA also has a dose-dependent effect, which can be optimized to the experimental model at hand. Nevertheless, there are several limitations to this model (Zheng et al., 2011). MTLE is a chronic neurodegenerative disorder which progresses over time; KA-induced neurogeneration on the other hand is an acute monophasic event. This fact cannot be mitigated by applying smaller doses of KA over an extended period of time, since this technique fails to mimic MTLE pathology adequately. Furthermore, kainate receptors are tetrameric ion channels consisting of 5 potential subunits – $GluR_5$, $GluR_6$, $GluR_7$, KA1, and KA2 – in various permutations; these subunits are expressed heterogeneously in various brain regions, and hence it is difficult to isolate or ascertain the effects imposed on any particular kainate receptor subtype (Vincent and Mulle, 2009). Likewise, the genetic backgrounds of various animal models lead to varying degrees of resistance to KA toxicity (Mckhann et al., 2003). To mitigate these confounding factors, we have restricted the use of our KA model to the well-characterized astrocytoma cell line U-373 (Volknandt et al., 2002), and Sprague Dawley rat hippocampus-derived primary neuron and astrocyte cultures.

1.11. ***Prior work leading up to this project*****:** A recent study by our lab group (Kodam et al., 2017) characterized the alterations in astrocyte APP and Aβ production/degradation that result from KA treatment by evaluating time-dependent alterations in the levels and cellular distribution of APP and its processing enzymes in the hippocampus of KA-treated rats. In parallel, the study also assessed the activity of β- and γ-secretase enzymes, as well as the levels of their products, the APP-CTFs and Aβ-related peptides to establish whether APP processing in KA-treated animals is altered towards the increased production of Aβ peptides.

Collectively, the results of this study reveal that KA administration increased the endogenous levels of Aβ peptides in rat hippocampi in addition to triggering seizures and neuronal loss. The presence of these peptides suggests that they may have a role in triggering neuronal loss in the KA model of TLE. Thus, defining the potential signaling pathways that underlie the degeneration of neurons following KA-induced seizure may provide a better understanding of the pathology associated with MTLE. However, changes in APP processing, Aβ synthesis, and its possible downstream consequences have thus far not been evaluated in astrocytes.

1.12. ***Hypothesis and objectives*****:** On the basis of earlier literature, as well as data obtained from our lab, we hypothesize that Aβ peptides derived from reactive astrocytes may have an important role in the degeneration of neurons in the KA model of epilepsy. To address this hypothesis, we will:

i) **Characterize the effect of KA on the expression and processing of APP and its proteolytic products in astrocyte cells**

ii) **Define the functional significance, if any, of astrocyte-derived Aβ on the viability of hippocampal neurons.**

Collectively, these experiments will provide an understanding of the potential amyloid-related mechanisms involved in KA. Establishing the significance of astrocyte-derived Aβ peptides, derived from reactive astrocytes, will provide an underlying basis for a role of these peptides in the loss of neurons in MTLE. Elucidating this connection has implications for both MTLE and AD, and may lead to new avenues of medical research and therapeutic development.

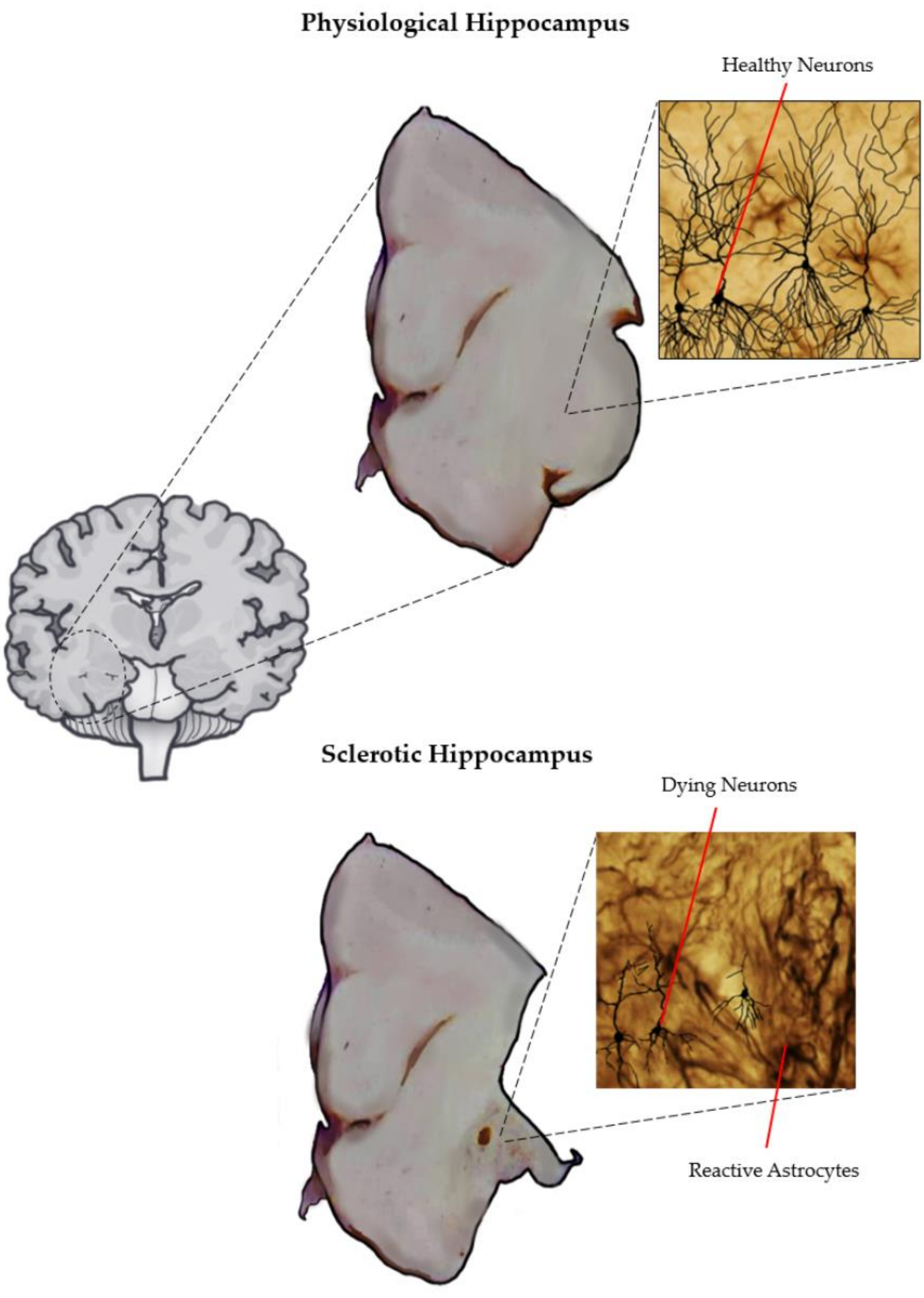


Figure 1.1: Graphic depiction of healthy and sclerotic hippocampi. Hippocampal sclerosis is highlighted by gross atrophy, neuronal death, and the proliferation of reactive astrocytes.

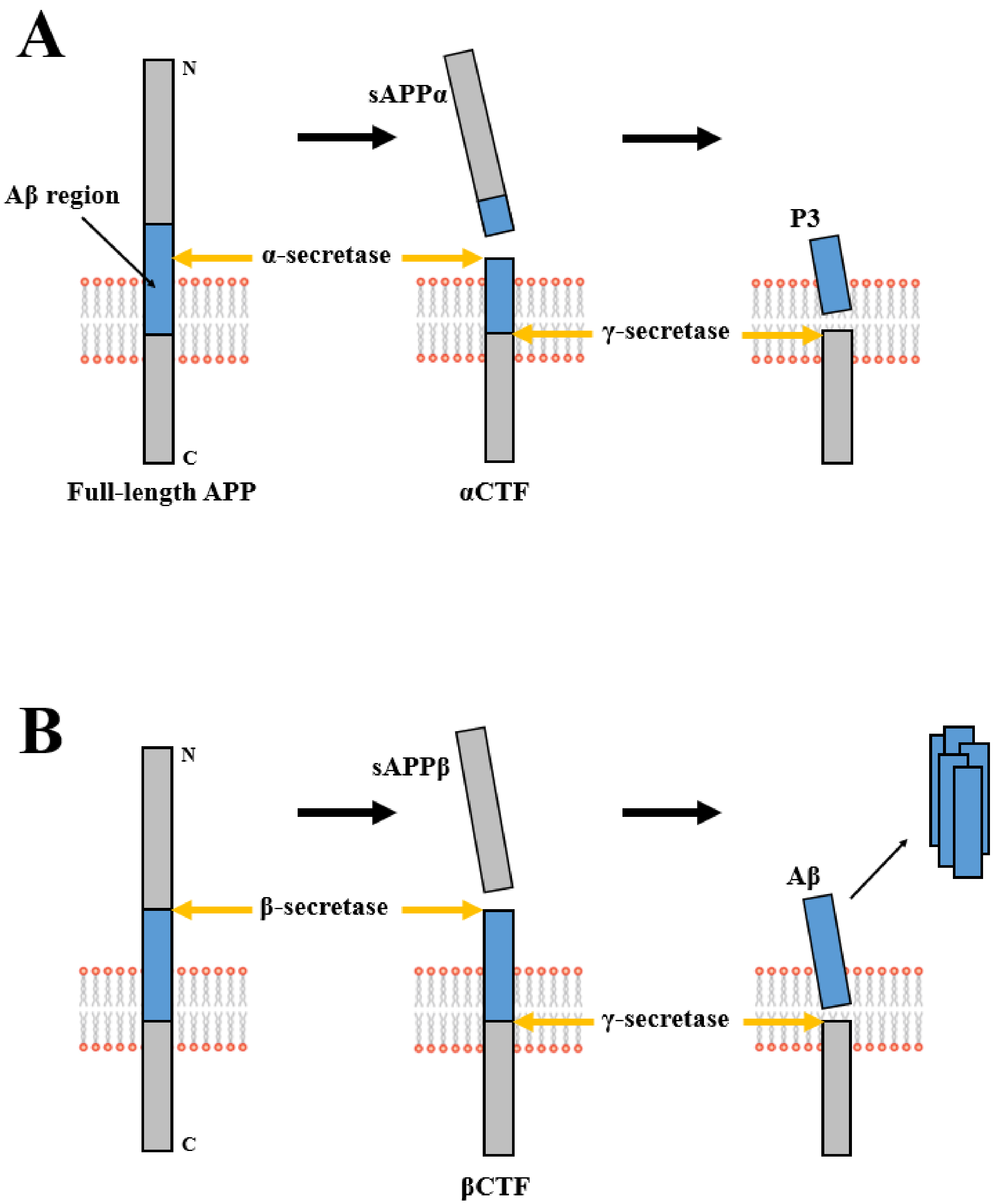


Figure 1.2: Main steps and enzymes involved in APP processing via the α-secretase (A) and β-secretase (B) pathways. Note that only the β-secretase pathway results in the production of full-length Aβ.

2. Materials and Methods

2.1. ***Reagents*****:** U-373 MG human astrocytoma cells (ATCC HTB 17) were obtained from American Type Culture Collection (Rockville, MD, USA). Rat hippocampal primary astrocytes and associated media components were acquired from ScienCell (Carlsbad, CA, USA), while all other cell culture reagents, including Dubecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS), Hank's balanced salt solution, Neurobasal media, B27 and N2 supplements, and TrypLE Express were purchased from Invitrogen (Burlington, ON, Canada). Also from Invitrogen were the NuPAGE electrophoresis (4-12%) Bis-Tris gels and the ELISA kits for the detection of rat $A\beta_{1-40}$ and $A\beta_{1-42}$. High sensitivity ELISA kits for the detection of human $A\beta_{1-40}$ and $A\beta_{1-42}$, were purchased from Millipore (Etobicoke, ON, Canada), along with Amicon Ultra-4 centrifugal filter columns. The enhanced chemiluminescence kit (ECL) and the bicinchoninic acid (BCA) protein assay kits were from Thermo Fisher Scientific (Montreal, QC, Canada). Cycloheximide, KA, γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and 3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Oakville, ON, Canada), kainate receptor antagonist (S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione (ACET) and AMPA-R antagonist 1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride (GYKI-54266) were from Tocris Bioscience (Bristol, United Kingdom), whereas the NMDA receptor antagonist 2-amino-5-phosphopentanoic acid (D-AP5) and β-secretase activity assay kit were from Abcam (Cambridge, United Kingdom). Protease inhibitor cocktail, BACE1 inhibitor BIV and fluorogenic γ-secretase substrate were from Calbiochem (San Diego, CA, USA). Sources of all primary antibodies used in the study are listed in Table 2.1. The associated horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz (Pasa Robles, CA, USA). All other reagents were from either Sigma-Aldrich or Fisher Scientific.

2.2. ***U373 human astrocytoma culturing and treatment paradigms*****:** U-373 MG human astrocytoma cells were cultured in DMEM with 10% heat-inactivated FBS and maintained at 37°C and 5% CO_2/air-humidified incubator. Cells were seeded at $1x10^4$ cells/cm^2, and the medium was replaced every 2-3 days. For experiments, cells were plated on 6-well plates at $1x10^4$ cells/cm^2 for 24hrs and grown to confluency. Media was replaced prior treatment with any reagents. In a series of experiments, U373 cells were first treated with different concentrations (10µM, 100µM, 1mM) of KA over a range of time-points (3, 6, 12, 24, and 48hrs). Following this protocol, we ascertained that 100µM KA for 24hr treatment is the most optimal condition and hence this concentration of KA was used in all subsequent experiments. In some experiments, U373 cells were first treated with either 100µM KA or saline, and then exposed to 30µg/ml cycloheximide for 0.5h, 1h, 2h, or 4h (Leem et al., 2002). In parallel, U373 cells were treated with or without 100µM KA for 24hr in the presence or absence of KA receptor antagonist ACET (100µM), NMDA receptor antagonist D-AP5 (100µM) or AMPA receptor antagonist GYKI-52466 (20µM). The concentrations used for ACET, D-AP5 and GYKI-52466 were based on previous results (Dargan et al., 2009, Choi et al., 1988, Paternain et al., 1995). After various experiments, cells were harvested and either used immediately or stored at -80°C until further processing.

2.3. *Primary rat hippocampal astrocyte cultures and treatment*: Primary rat hippocampal astrocytes (ScienCell, CA, USA) were seeded on Poly-D-lysine-coated plates and grown in the accompanying astrocyte media at 37°C as per the manufacturer's instructions. Cells were grown to confluency and passaged using TrypLE Express. For experiments, cells were plated on 6-well plates at $1x10^4$ cells/cm^2 for 24hrs and grown to confluency. Media was replaced prior treatment with any drug/substance. In a series of experiments, cultured primary astrocytes were treated with 100µM KA for different periods of time (3, 6, 12, 24, and 48hrs) and then processed for Western blotting, ELISA or cell viability assays. In parallel, cultured astrocytes were treated with 100µM KA for 24hr in the presence or absence of 20µM DAPT (inhibitor of γ-secretase activity) and the cells were processed for ELISA to detect cellular and secretory Aβ levels.

2.4. *Primary rat hippocampal neuronal and astrocyte/neuronal co-culture and treatment*: Primary rat hippocampal neuronal cultures were prepared from 17/18-day-old embryos of time-pregnant Sprague-Dawley rats (Charles River, Quebec, Canada) as described previously (Zheng et al., 2002; Wei et al., 2008). All animal protocols were in accordance with the Institutional and Canadian Council on Animal Care guidelines. In brief, the hippocampal region was dissected in Hanks balanced salt solution supplemented with 100µM HEPES, 10mM Na-pyruvate, 50µM L-glutamine, 10U/ml penicillin and 10mg/ml streptomycin and digested with 0.25% trypsin/EDTA.

The cell suspension was filtered through a cell strainer and then plated on 6-well plates. The cultures were grown at 37°C in a 5% CO_2-humidified atmosphere in Neurobasal medium supplemented with B27, 50μM L-glutamine, 100μM HEPES, 10mM Na-pyruvate, 10U/ml penicillin and 10mg/ml streptomycin, and 1% FBS. The medium was replaced 1day later without FBS and all experiments were performed on day 6 after plating. To prepare astrocyte-neuronal co-cultures, hippocampal neurons were prepared as described above, and then seeded directly on a layer of astrocytes grown to confluency. Media conditions for co-cultures follow the protocol for neuronal culture and all experiments were performed 6 days following cell plating. To determine the effect of KA treatment on cell survival, pure neuronal cultures and mixed primary neuron/astrocyte cocultures were exposed to a range of KA concentrations (50-200μM) over the course of 24 or 48hrs with or without 20μM DAPT. In all cases, cell viability was assessed via MTT assay (Maulik et al., 2015).

2.5. *Western blotting*: U373 and rat primary astrocyte cultured cells from different experimental paradigms were lysed with radioimmunoprecipitation assay (RIPA) buffer, and the protein contents were quantified with a BCA kit. Samples were then denatured and resolved by either 7-17% gradient polyacrylamide gels made in house, or premade NuPAGE 4-12% Bis-Tris gels from Invitrogen. Following electrophoresis, proteins were transferred to nitrocellulose membranes, blocked with 5% milk and incubated overnight at 4°C with anti-APP (22C11), anti-CTF (Y188), anti-ADAM10, anti-BACE1, anti-nicastrin, anti-PEN2, anti-APH1, anti-PS1, anti-IDE or anti-neprilysin antisera. Following day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) and immunoreactive proteins were detected with enhanced chemiluminescence. All blots were re-probed with anti-β-actin antibody as a loading control and quantified using a MCID image analysis system as described earlier (Wang et al., 2015).

2.6. *β- and γ-secretase activity assays*: Control and KA-treated cultured U373 astrocytes were homogenized with RIPA buffer, centrifuged at 10,000*g* for 5min and then processed to measure β-secretase BACE1 activity according to the manufacturer's instructions as described earlier (Maulik et al., 2015). The fluorescence was measured at excitation wave length of 355 nm and emission wave length of 495nm. The γ-secretase activity was measured on crude membrane fractions. Briefly, control and treated cells were lysed using sample buffer (10mM Tris base, 1mM

EDTA, 1x Protease Inhibitor Cocktail, pH 7.4) and then centrifuged to remove nuclei and cell debris. The supernatant was further centrifuged at 100,000*g* to separate the membrane fraction which was solubilized and its protein content was determined using BCA. The γ-secretase activity was then measured in 50mM Tris-HCl (pH 6.8), 2mM EDTA and 0.25% CHAPSO with 8μM fluorogenic γ-secretase substrate in 50μg protein as described earlier (Maulik et al., 2015). The fluorescence was detected at excitation wavelength of 355nm and emission wavelength of 440nm and the specificity was determined by incubating samples with 100μM γ-secretase inhibitor L-658,458. All samples were assayed in duplicate and results were obtained from four independent experiments**.**

2.7. ***ELISA for $A\beta_{1\text{-}40/1\text{-}42}$***: To measure cellular Aβ levels, KA-treated cultured and control U373 cells were solubilized in RIPA buffer, centrifuged, and then assayed for human $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ using respective human ELISA kits as described earlier (Wang et al., 2015). For the measurement of secreted $A\beta_{1\text{-}40/1\text{-}42}$ peptides, conditioned media collected from control and KA-treated cells were concentrated using Amicon filtration columns with 3kDa MW cut-off and then analyzed using the ELISA kits. The absorbance was read with a microplate reader, and the amount of $A\beta_{1\text{-}40/1\text{-}42}$ in each sample was calculated from the standard curve. The levels of cellular and secreted $A\beta_{1\text{-}40/1\text{-}42}$ peptides in rat primary astrocyte cells treated with either saline, 100μM KA with or without 20μM DAPT were also similarly measured with commercial rat/mouse ELISA kits as per the manufacturer's instructions. All samples were assayed in duplicate and results presented were obtained from four independent experiments.

2.8. ***Cell viability assays***: The viability of cultured U373 cells, primary rat neurons, astrocytes, or mixed neuron/astrocyte cultures exposed to various treatment paradigms outlined above was assessed using the cell proliferation colorimetric MTT assay as described earlier (Maulik et al., 2015). In brief, control and treated culture plates were replaced with new medium containing 0.25% MTT and then incubated for 4hr at 37°C. The reaction was terminated and measured spectrophotometrically at 570nm. All experiments were performed in quadruplicate and results presented were obtained from four independent experiments.

2.9. ***Statistical analysis***: All data were collected from four biological repeats and expressed as means ± SEM. In instances of two independent mean comparisons, Student's t-test was used. In instances of multiple mean comparisons, ANOVA was used, followed by Bonferroni's *post hoc* analysis. A *p* value under 0.05 was accepted as statistically significant. All statistical analysis were performed using GraphPad Prism (GraphPad Software, Inc., CA, USA).

Table 2.1: Details of the primary antibodies used in this study

ANTIBODY NAME	TYPE	Western Dilution	SOURCE
Anti-Amyloid Precursor Protein (22c11)	Monoclonal	1-3000	EMD Millipore
Anti-Amyloid Precursor Protein (Y188)	Monoclonal	1-3000	Abcam (Cambridge, MA)
Anti-Beta site APP Cleaving Enzyme 1 (BACE1)	Polyclonal	1-2000	Chemicon Intl. (Temecula, CA)
Anti-A Disintegrin and metalloproteinase domain-containing protein 10	Polyclonal	1-2000	EMD Millipore
Anti-Presenilin 1 (PS1)	Polyclonal	1-2000	Dr. G. Thinakaran[1]
Anti-Nicastrin	Polyclonal	1-800	Santa Cruz
Anti-APH1	Polyclonal	1-800	Dr. G. Thinakaran[1]
Anti-Presenilin Enhancer Homolog 2 (PEN2)	Polyclonal	1-2000	Dr. G. Thinakaran[1]
Anti-Insulin Degrading Enzyme	Polyclonal	1-500	Abcam (Cambridge, MA)
Anti-Neprilysin	Monoclonal	1-500	Abcam (Cambridge, MA)
Anti-Glial Fibrillary Acidic Protein (GFAP)	Polyclonal	1-5000	Sigma (Oakville, ON)
Anti-β-actin	Polyclonal	1-5000	Sigma (Oakville, ON)

[1]The University of Chicago, IL, USA

3. Results

3.1. ***Effects of kainic acid on APP metabolism in U-373 cells*:** U-373 MG is a well characterized human glioblastoma cell line enriched with GFAP-positive astrocytes which have been used extensively for various experimental paradigms (Volknandt et al., 2002). To characterize the potential effects of KA on APP metabolism in astrocytes, we first evaluated APP holoprotein levels in U373 cell following exposure to different concentrations (10μM, 100μM and 1mM) of KA for varying lengths (3h - 48h) of time. Our results revealed a significant time-dependent increase followed by decline in APP levels as a consequence of treatment with different doses of KA (Fig. 3.1). A significant increase in APP level was evident at 3h with 10μM KA, 6-24hr with 100μM KA and 12-24hr with 1mM KA. The upregulation of APP was most prominent at 24h following treatment with 1mM KA, reaching ~250% of the control levels before declining by 48h of treatment (Fig. 3.1C). Since 100μM KA showed a protracted increase in APP levels over 6-24hr post-treatment (Fig. 3.1B), this dose of KA was used in all subsequent experiments. In parallel to APP, we analysed the levels of α- and β-secretase cleavage products (α-CTF and β-CTF, respectively) following exposure to KA for different periods of time (Fig. 3.2). Our results indicate that 100μM KA induces a parallel increase in both α-CTF and β-CTF levels between 6-24hr post-treatment. Although the levels of both CTFs increased following KA treatment, the magnitude of change was more prominent for β-CTF (Fig. 3.2B) compared to α-CTF (Fig. 3.2A).

To assess whether altered levels of α-/β-CTFs are the consequence of differential APP processing, we first evaluated steady state levels of α-secretase ADAM10 (Fig. 3.3A), β-secretase BACE1 (Fig. 3.3B) and the components of the γ-secretase complex (Fig. 3.4) in U373 cells following exposure to 100μM KA over 3-4hr periods. Interestingly, our results revealed no significant changes in ADAM10, BACE1, or any of the four components of the γ-secretase complex (PS1, nicastrin, APH1 or PEN2) at any time point. Considering the evidence that steady-state levels of BACE1 or γ-secretase complex does not necessarily correspond with enzyme activity (Ourdev et al., 2015; Thinakaran et al., 1997), we subsequently assessed changes in the activity of these three secretases (Fig. 3.5). While no observable change was apparent in the activity of either ADAM10 (Fig. 3.5A) or BACE1 (Fig. 3.5B) in cells treated with 100μM KA for 24h, the activity of γ-secretase increased three-fold in KA-treated cells compared to control cells (Fig. 3.5C), suggesting that KA may lead to an increased production of Aβ-related peptides, at least in part, by modulating the activity of this crucial enzyme. To determine whether decreased turnover may also contribute to the enhanced levels of the peptides, cultured U373 cells were treated with or without 100mM KA for 24hr and then exposed to cycloheximide for different periods of time (i.e., 0, 0.5h, 1h, 2h and 4h) to inhibit *de novo* synthesis of proteins including APP (Leem et al., 2002). As expected, the levels of APP and α-CTF/β-CTF were markedly higher in U373 cells treated with cycloheximide, but these peptides decline as a function of time without any significant difference in rate between KA-treated and untreated control cells (Fig. 3.6). We also found the steady state levels of the two main APP clearance peptides, IDE and neprilysin, unchanged in response to KA treatment (Fig. 3.7).

3.2. *Effects of kainic acid on Aβ levels/secretion in U-373 cells*: To determine whether the KA-induced altered APP metabolism can lead to increased levels/secretion of Aβ peptides, we measured $A\beta_{1-40}$ and $A\beta_{1-42}$ levels using ELISA in both cell lysates and conditioned media of U-373 cells treated with or without 100μM KA for 24h. Our results indicate that the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ are markedly increased in both cell lysates and conditioned media following KA treatment, suggesting increased production and secretion of these peptides (Fig 3.8). Since KA treatment does not have any apparent toxicity to astrocytes (Fig. 3.9), as assessed by MTT assay, we can conclude that these changes in APP metabolism were not due to cell loss between experimental groups.

3.3. *Effects of kainic acid antagonist ACET on APP metabolism*: To determine if the effects of KA on APP metabolism are mediated by selective activation of the KA receptors, we used the antagonist ACET which has previously shown to block the response of KA (Dargan et al., 2009). In our paradigm, U373 cells were treated with 100μM KA in the presence or absence of 100μM ACET for 24h and then cells were processed to evaluate the levels of APP and its cleaved products. While ACET itself did not alter the levels of either APP or α-CTF/β-CTF, it significantly attenuated the effects of KA on APP holoprotein (Figure 3.10A) and its metabolites (Fig. 3.10), including α-CTF (Fig. 3.10B), β-CTF (Fig. 3.10C) and both cellular and secreted forms of Aβ peptides (Figure 3.11). In contrast to ACET, neither the NMDA receptor antagonist D-AP5 (Fig. 3.12) nor the AMPA receptor antagonist GYKI-54266 (Fig. 3.13) was able to attenuate the effects of KA on the levels of APP or its metabolites.

3.4. ***Effects of kainic acid on APP metabolism in rat primary astrocytes*****:** To confirm that the effects of KA observed in our glioblastoma U373 cells occur in primary astrocytes, rat hippocampal astrocytes were treated with 100μM KA for different periods of time (3-48hrs) and then assessed to measure the levels of APP holoprotein and its metabolites. Similar to our results with U373 cells, the levels of APP (Fig. 3.14A), α-CTF (Fig. 3.14B) as well as β-CTF (Fig. 3.14C) were markedly increased between 12-24hr in KA treated astrocytes compared to control astrocytes. Additionally, we did not observe any alteration in the steady state levels of either BACE1 (Fig. 3.15A) or PS1 (Fig 3.15B) at any time point following KA treatment.

3.5. ***Effects of kainic acid on Aβ levels/secretion in rat primary astrocytes*****:** To determine whether changes in the levels of APP and CTFs can lead to higher levels of Aβ peptides, rat primary astrocytes were treated with 100μM KA for 12hr, 24, and 48hrs and then processed to measure cellular/secretory Aβ levels using ELISA. Our results showed that cellular levels of $A\beta_{1-40}$ were significantly increased at 12hr and 24hr, whereas $A\beta_{1-42}$ level were increased only at 12hr after KA treatment (Fig. 3.16A). The secretory levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, on the other hand, were markedly increased with time following exposure to 100μM KA (Fig. 3.16B). Pre-treatment with the γ-secretase inhibitor DAPT attenuated enhanced levels of Aβ peptides, suggesting that an increased production/secretion of the peptides is possibly triggered by KA treatment (Fig. 3.16C). In parallel, we observed that the viability of cultured astrocytes was not compromised after 48hr exposure to either 100μM KA, DAPT, or a combination of the two (Fig. 3.17A), suggesting once again that these changes were not due to cell loss.

3.6. ***Kainic acid-induced neurodegeneration and its attenuation by γ-secretase inhibitor*****:** To define the functional significance of astrocyte-derived Aβ peptides in KA-induced toxicity, pure rat hippocampal cultured neurons were exposed to a range of KA doses (50, 100, and 200μM) for either 24 or 48hrs, and their cell viability was assessed using an MTT assay. As expected, viability of cells was reduced significantly in most conditions, but it did not exhibit any variation with either increasing concentrations or time following exposure to KA (Fig. 3.17B). Interestingly, treating mixed neuron/astrocyte co-cultures with 100μM KA for 24hrs was found to reduce cell viability significantly more than what was observed with pure neuronal cultures (Fig. 3.18A) At the same time, we observed that the viability of cultured astrocytes was not compromised after 48hr exposure to either 100μM KA or 20μM DAPT (Fig. 3.17A). Since KA does not appear to induce astrocyte death, we sought to determine whether the added neuronal loss observed in mixed co-cultures could be due to the presence of Aβ peptides generated by the cultured astrocytes as a result of KA treatment. To this end, we pretreated mixed neuron/astrocyte co-cultures with or without 20μM DAPT, and then exposed them to 100μM KA for 24hrs. Our results clearly showed that DAPT treatment can ameliorate KA toxicity (Fig. 3.18B), thereby determining that astrocyte-derived Aβ-related peptides play a role in the degeneration of neurons.

Fig. 3.1: A-C; Histograms and associated representative western blot images of APP expression time-response to treatment with 10μM (A), 100μM (B) or 1mM (C) of KA in U373 cells. KA treatment resulted in a dose- and time-dependent increase in APP holoprotein levels, peaking between 12 and 24h post-treatment for 1mM, 6-24h for 100μM, and at 3h for 10μM of KA. Data represent means±SEM from 3-4 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

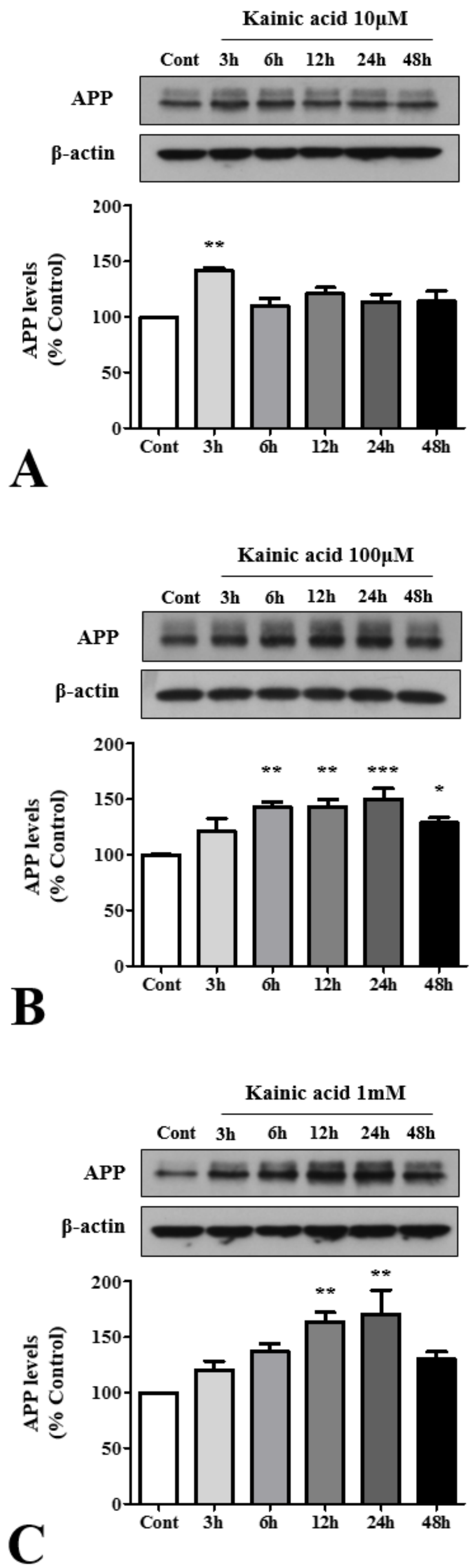

Kainic acid 10μM
Cont 3h 6h 12h 24h 48h
APP
β-actin
APP levels (% Control)
200
150
100
50
0
**
Cont 3h 6h 12h 24h 48h
A
Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
APP
β-actin
APP levels (% Control)
200
150
100
50
0
** ** *** *
Cont 3h 6h 12h 24h 48h
B
Kainic acid 1mM
Cont 3h 6h 12h 24h 48h
APP
β-actin
APP levels (% Control)
200
150
100
50
0
** **
Cont 3h 6h 12h 24h 48h
C

Fig. 3.2: A and B; Histograms and associated representative western blot images of CTF expression time-response to treatment with 100μM of KA in U373 cells. KA triggers a time-dependent increase in both α- (A) and β-CTFs (B). Data represent means±SEM from 3-4 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

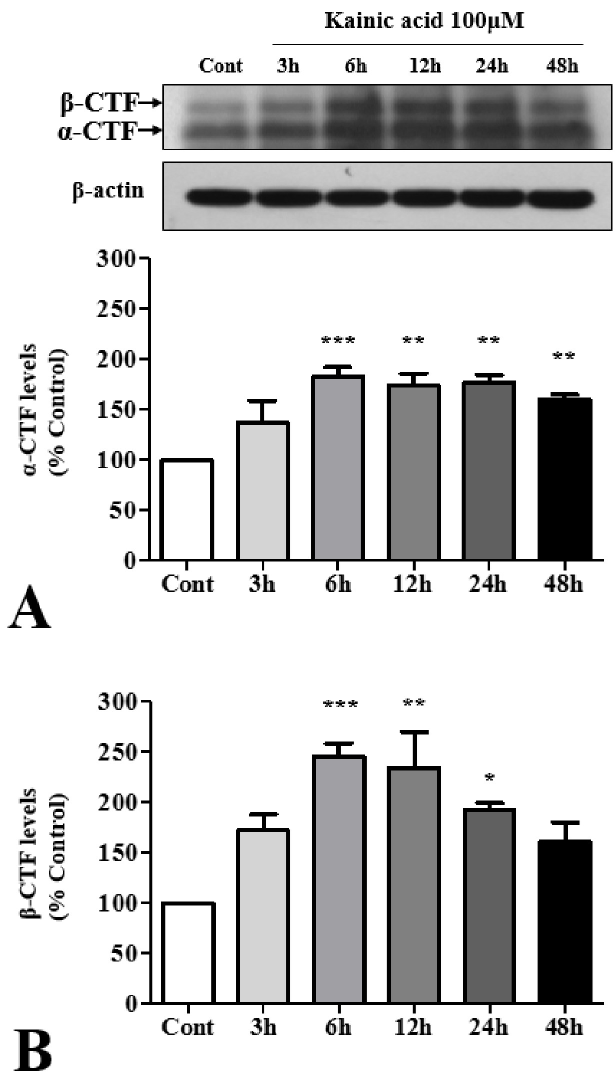
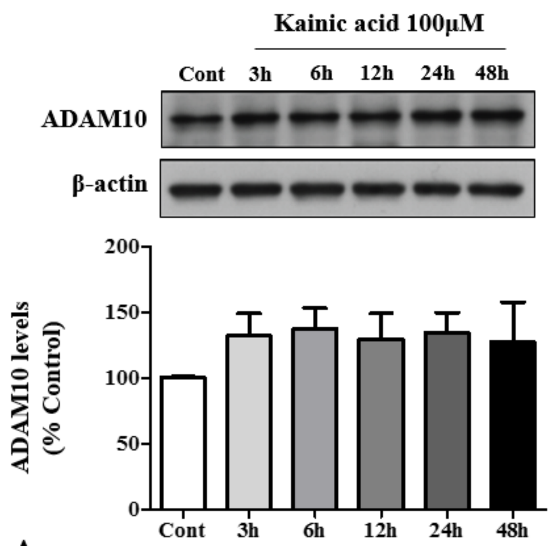
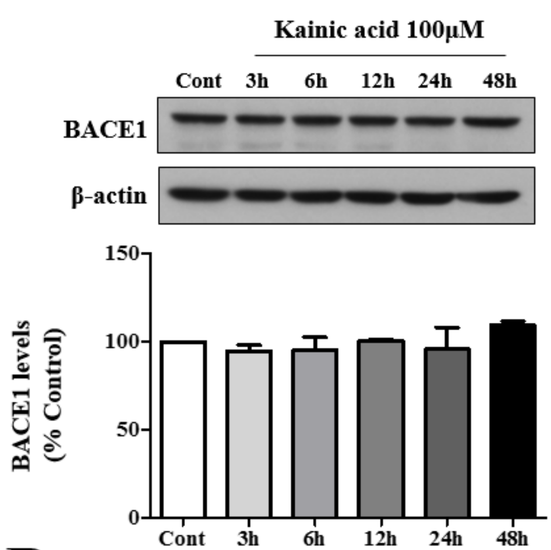

Kainic acid 100μM
Cont
3h
6h
12h
24h
48h
β-CTF
α-CTF
β-actin
α-CTF levels (% Control)
300
250
200
150
100
50
0
***
**
**
**
Cont
3h
6h
12h
24h
48h
A
β-CTF levels (% Control)
300
250
200
150
100
50
0
***
**
*
Cont
3h
6h
12h
24h
48h
B

Fig. 3.3: A and B; Histograms and associated representative western blot images of ADAM10 (A) and BACE1 (B) expression in response to treatment with 100μM of KA in U373 cells. KA was not found to cause any significant changes in the levels of either secretase enzyme. Data represent means±SEM from 3-4 independent experiments.



Kainic acid 100µM
Cont
3h
6h
12h
24h
48h
ADAM10
β-actin
ADAM10 levels (% Control)
200
150
100
50
0
Cont
3h
6h
12h
24h
48h

A



Kainic acid 100µM
Cont
3h
6h
12h
24h
48h
BACE1
β-actin
BACE1 levels (% Control)
150
100
50
0
Cont
3h
6h
12h
24h
48h

B

Fig. 3.4: A-D; Histograms showing the alteration of γ-secretase components PS1 (A), Nicastrin (B), APH1 (C), and PEN2 (D) in U373 cells following 100μM of KA over time. KA did not affect the levels of these proteins at any time-point. All data represent means±SEM from 3-4 independent experiments.

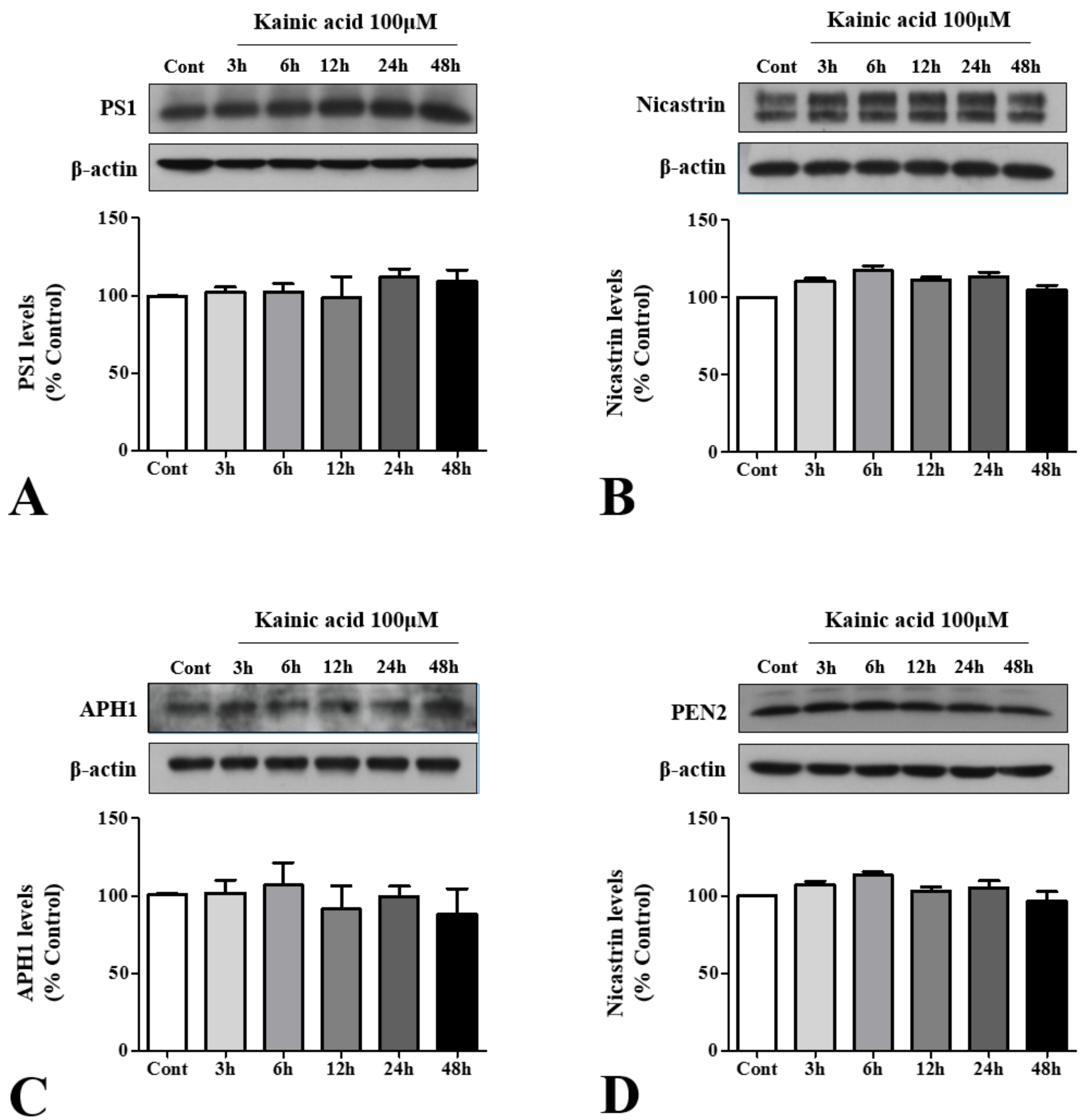
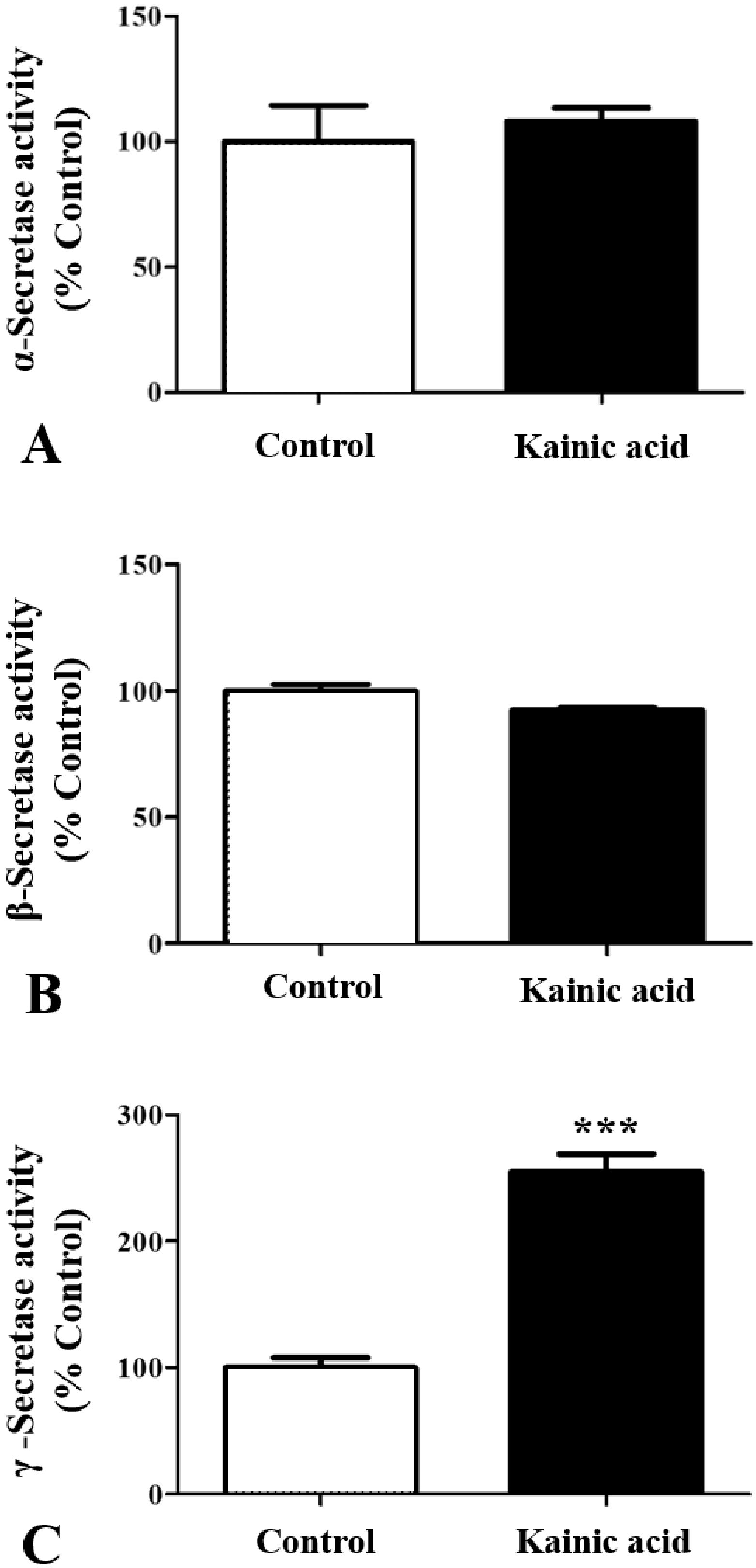

Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
PS1
β-actin
PS1 levels (% Control)
150 100 50 0
Cont 3h 6h 12h 24h 48h
A
Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
Nicastrin
β-actin
Nicastrin levels (% Control)
150 100 50 0
Cont 3h 6h 12h 24h 48h
B
Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
APH1
β-actin
APH1 levels (% Control)
150 100 50 0
Cont 3h 6h 12h 24h 48h
C
Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
PEN2
β-actin
Nicastrin levels (% Control)
150 100 50 0
Cont 3h 6h 12h 24h 48h
D

Fig. 3.5: A-C; Histograms depicting activities of α-secretase (A), β-secretase (B), and γ-secretase (C) in cultured U373 cells following 24h treatment with 100μM KA treatment. KA was found to trigger significant increases in γ-secretase. All data represent means±SEM from 3-4 independent experiments. ***$p<0.001$.


150
100
50
0
α-Secretase activity
(% Control)
A
Control
Kainic acid
150
100
50
0
β-Secretase activity
(% Control)
B
Control
Kainic acid
300
200
100
0
γ -Secretase activity
(% Control)
***
C
Control
Kainic acid

\

Fig. 3.6: Western blots and respective histograms of control and KA-treated U373 cells in the presence and absence of cycloheximide (cyclo) and probed for APP (A), α-CTF (B), and β-CTF (C). Densitometry analysis shows a time-dependent decrease in the levels of APP holoprotein, αCTF, and βCTF as result of KA treatment, but no overall difference between degradation rates in KA-treated vs control cells. All data represent means±SEM from 3-4 independent experiments.

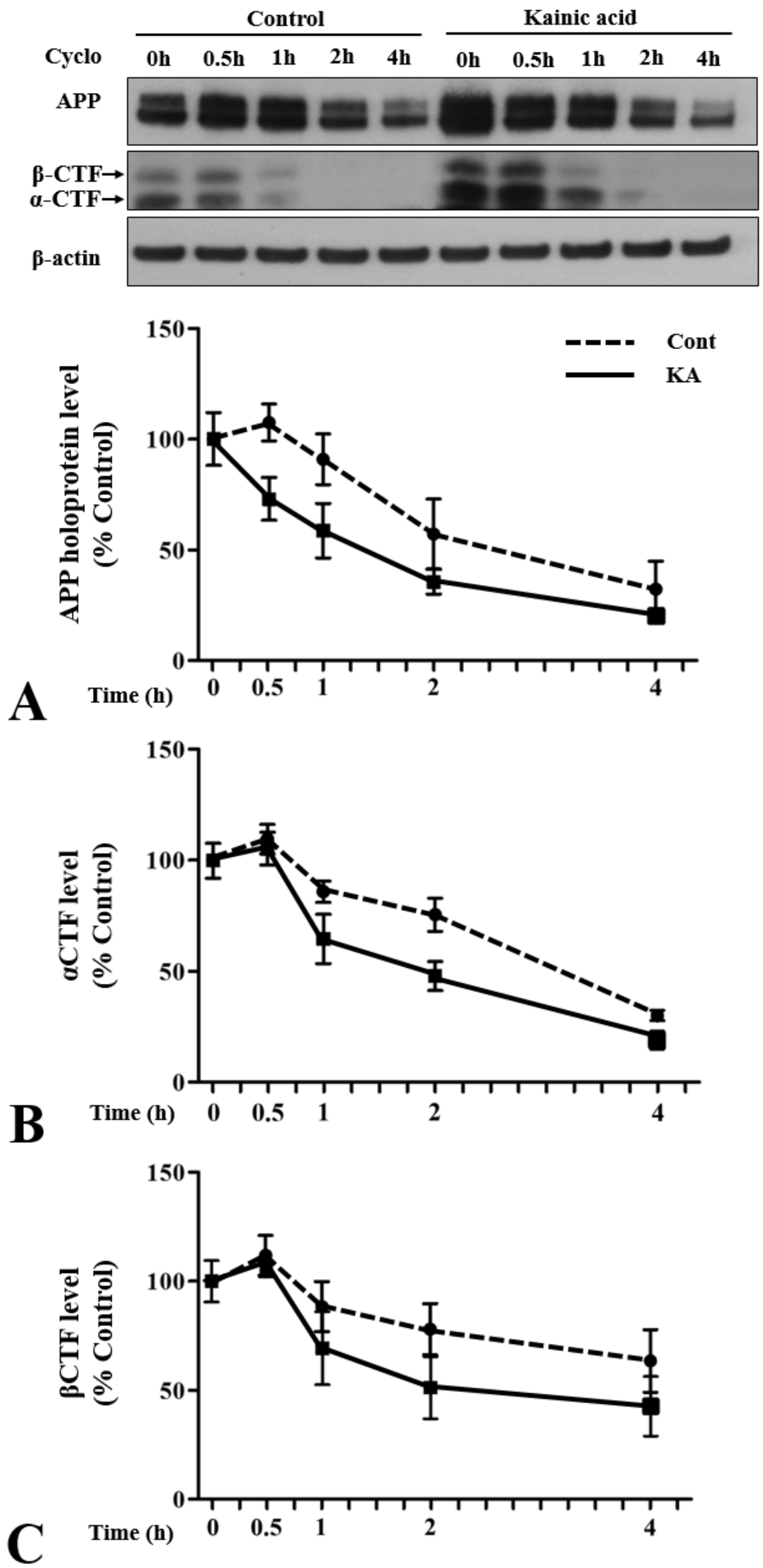

Control
Kainic acid
Cyclo
0h
0.5h
1h
2h
4h
0h
0.5h
1h
2h
4h
APP
β-CTF
α-CTF
β-actin
APP holoprotein level (% Control)
Cont
KA
Time (h)
A
αCTF level (% Control)
B
βCTF level (% Control)
C

Fig. 3.7: A and B; Histograms showing IDE (A) and Nephrilysin (B) levels in U373 cells treated with 100μM of KA over time. No alterations were observed in the expression levels of either protein in response to KA at any time point. All data represent means±SEM from 3-4 independent experiments.

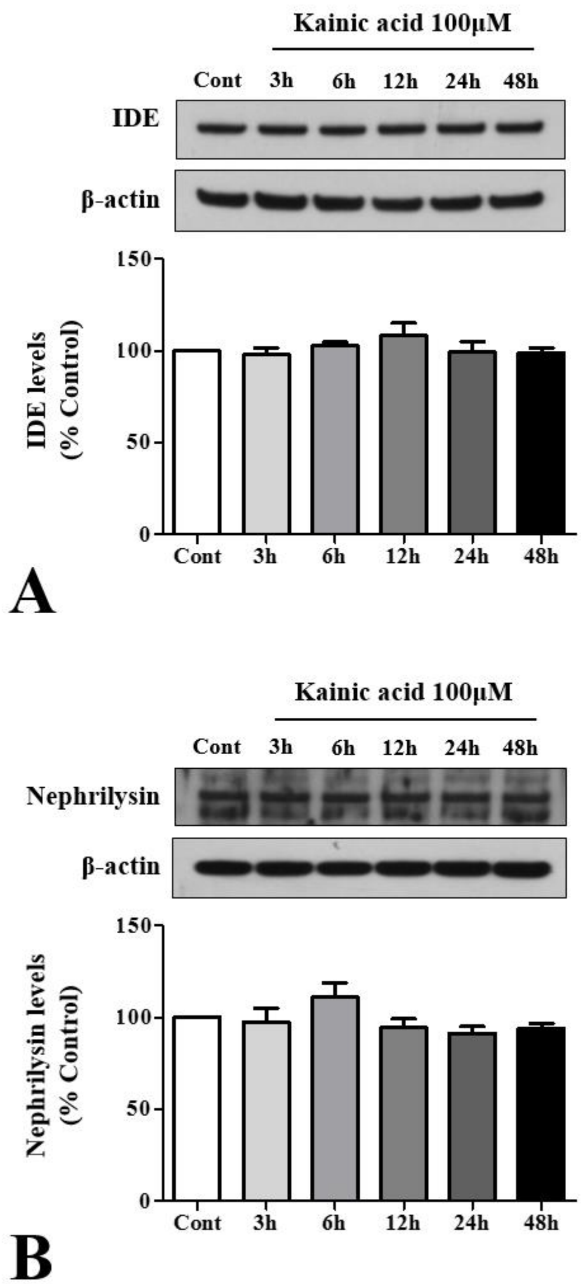
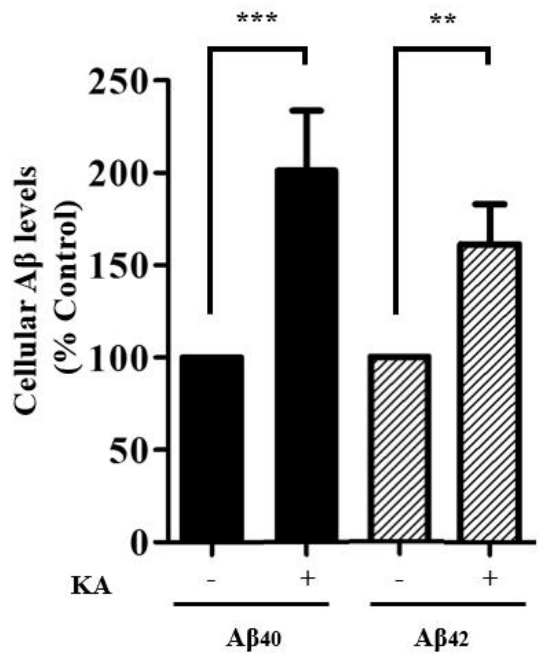
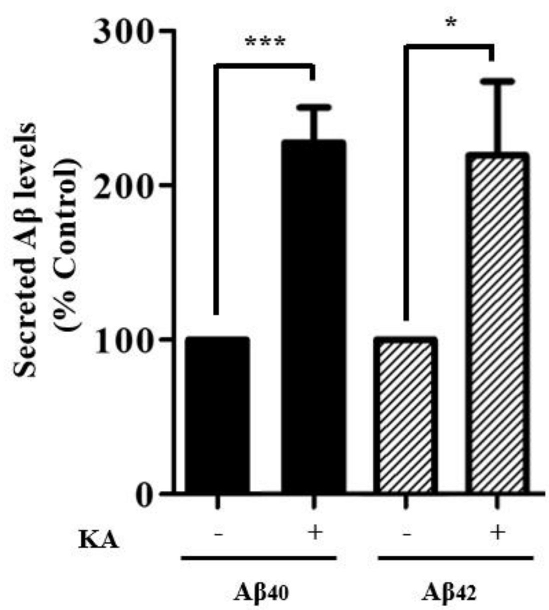

Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
IDE
β-actin
IDE levels
(% Control)
150
100
50
0
Cont 3h 6h 12h 24h 48h
A
Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
Nephrilysin
β-actin
Nephrilysin levels
(% Control)
150
100
50
0
Cont 3h 6h 12h 24h 48h
B

Fig. 3.8: A and B; Histograms showing the alteration in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in cell lysates (A) and media (B) of U373 cells with or without 100µM of KA treatment. Both $A\beta_{1-40}$ and $A\beta_{1-42}$ show a significant increase in the cell lysates as well as in the media following KA treatment. All data represent means±SEM from 3-4 independent experiments. *p<0.05, **p<0.01, ***p<0.001.


***
**
250
200
150
100
50
0
Cellular Aβ levels (% Control)
KA
- + - +
Aβ40
Aβ42

A


***
*
300
200
100
0
Secreted Aβ levels (% Control)
KA
- + - +
Aβ40
Aβ42

B

Fig. 3.9: Histogram showing no change in the viability of cultured U373 astrocytes following treatment with KA as measured by an MTT assay. All data represent means±SEM from 3-4 independent experiments.

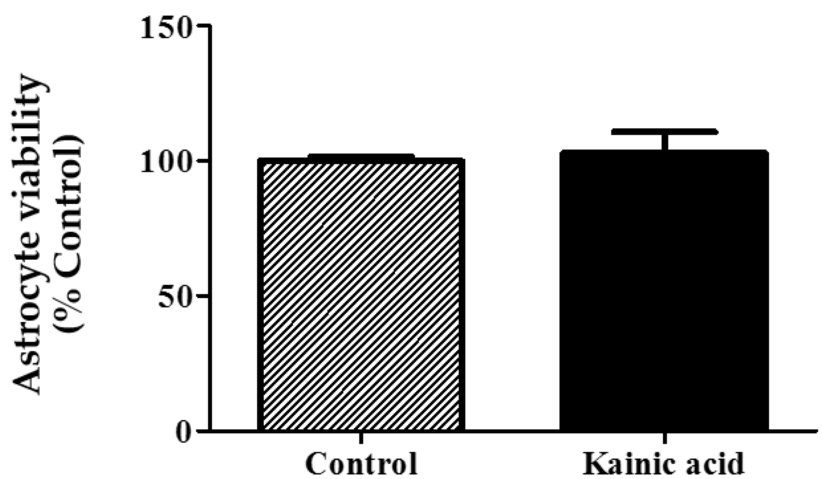

150
100
50
0
Astrocyte viability
(% Control)
Control
Kainic acid

Fig. 3.10: A-C; Histograms showing the alteration in APP (A), α-CTF (B), and β-CTF (C) levels in U373 cells treated for 24h with KA, ACET, or both, relative to control (Cont). ACET alone was not found to alter protein levels. At the same time, the increase in expression of APP and β-CTF protein species triggered by KA was found to be reversed by the application of the ACET inhibitor. All data represent means±SEM from 3-4 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns = not statistically significant.

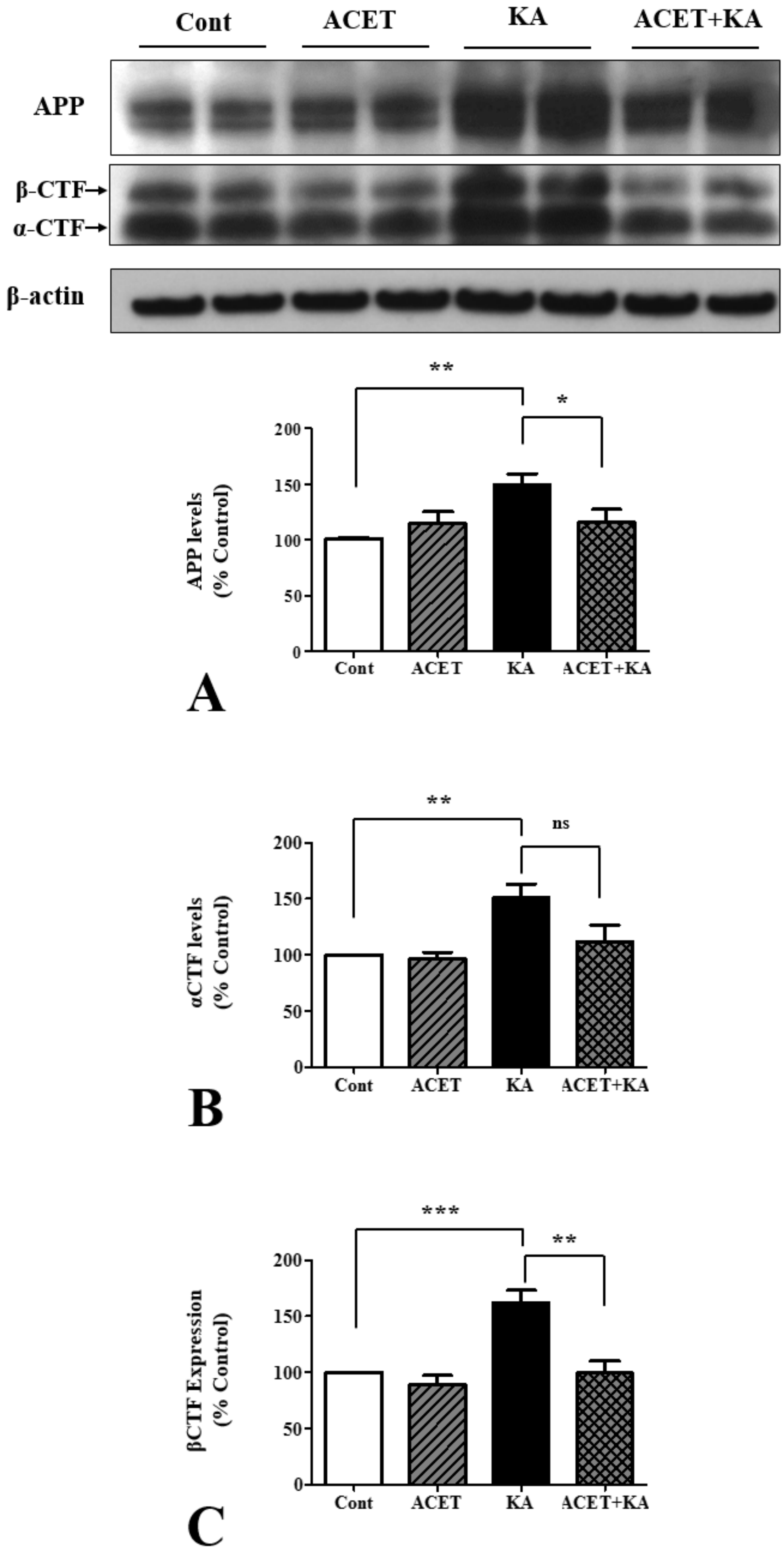

Cont
ACET
KA
ACET+KA
APP
β-CTF
α-CTF
β-actin
**
*
APP levels (% Control)
200
150
100
50
0
Cont
ACET
KA
ACET+KA
A
**
ns
αCTF levels (% Control)
200
150
100
50
0
Cont
ACET
KA
ACET+KA
B
***
**
βCTF Expression (% Control)
200
150
100
50
0
Cont
ACET
KA
ACET+KA
C

Fig. 3.11: A and B; Histograms showing the alteration in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in cell lysates (A) and media (B) of U373 cells in the presence or absence of 100μM of KA and/or ACET. Both $A\beta_{1-40}$ and $A\beta_{1-42}$ show a significant increase in the cell lysates as well as in the media following KA treatment. Although ACET alone does not appear to change Aβ levels relative to control, it effectively nullifies any changes in both cellular and secretory Aβ peptides induced by KA. All data represent means±SEM from 3-4 independent experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$.

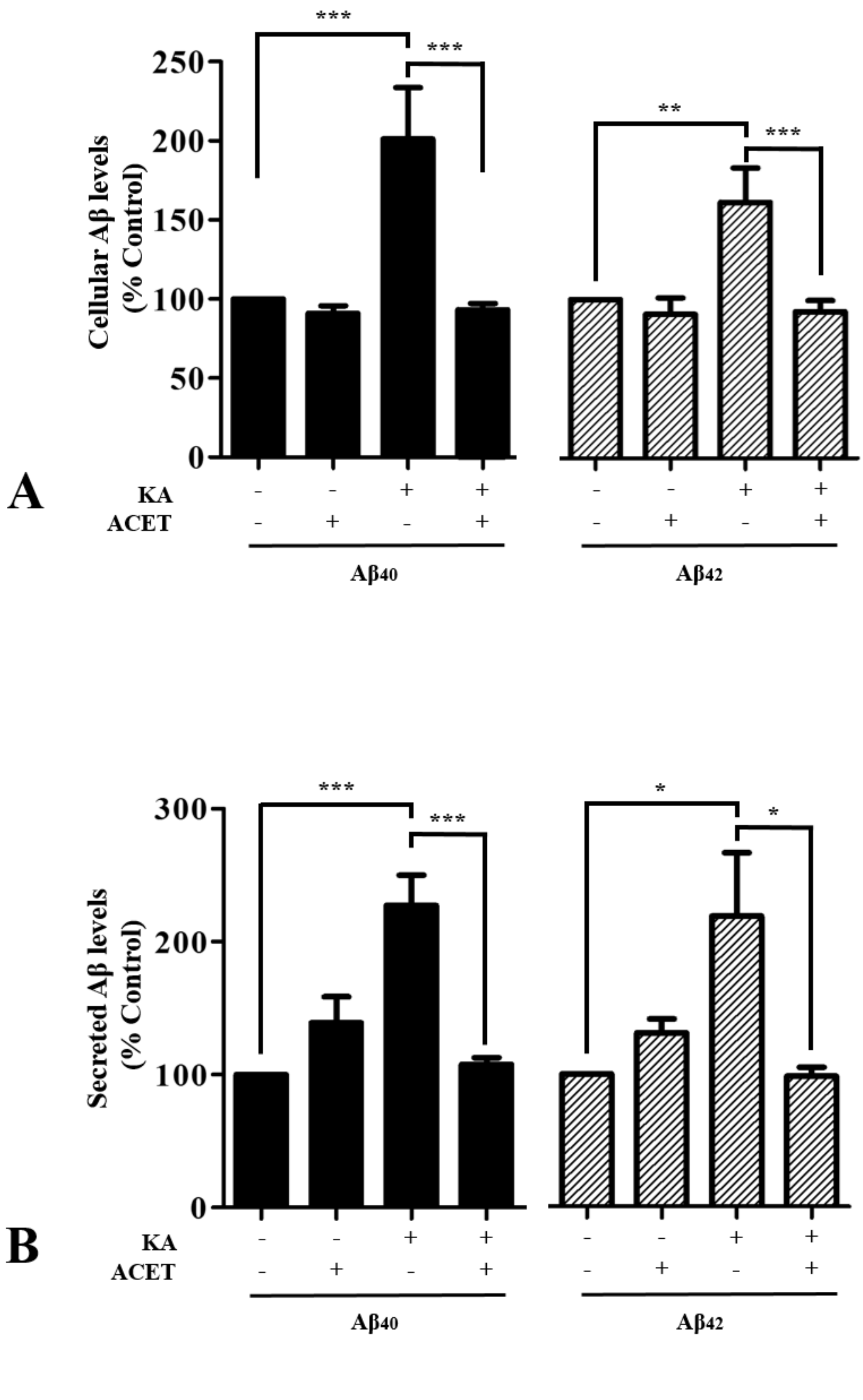

***
***
250
200
150
100
50
0
Cellular Aβ levels (% Control)
**
***
A
KA
ACET
- - + +
- + - +
Aβ40
Aβ42
***
***
300
200
100
0
Secreted Aβ levels (% Control)
*
*
B
KA
ACET
Aβ40
Aβ42

Fig. 3.12: A-C; Histograms showing the alteration in APP (A), α-CTF (B), and β-CTF (C) levels in U373 cells treated for 24h with KA, AP5, or both, relative to control (Cont). AP5 alone was not found to alter protein levels. At the same time, AP5 was also unable to affect changes in the expression of all three protein species triggered by KA. All data represent means±SEM from 3-4 independent experiments. **p<0.01, ***p<0.001, ns = not statistically significant.

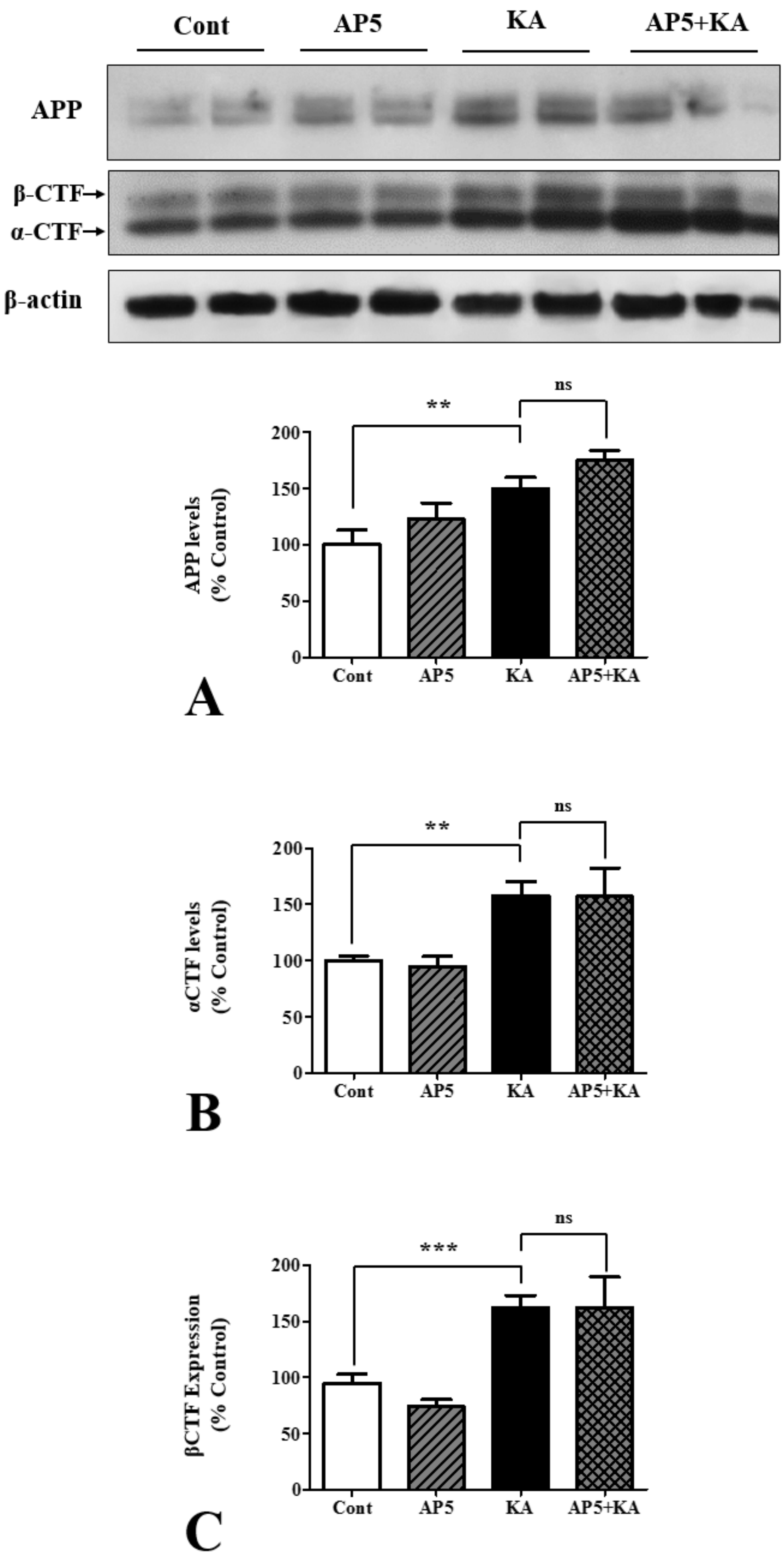

Cont
AP5
KA
AP5+KA
APP
β-CTF
α-CTF
β-actin
**
ns
APP levels
(% Control)
200
150
100
50
0
Cont
AP5
KA
AP5+KA
A
**
ns
αCTF levels
(% Control)
200
150
100
50
0
Cont
AP5
KA
AP5+KA
B
***
ns
βCTF Expression
(% Control)
200
150
100
50
0
Cont
AP5
KA
AP5+KA
C

Fig. 3.13: A-C; Histograms showing the alteration in APP (A), α-CTF (B), and β-CTF (C) levels in U373 cells treated for 24h with KA, GYKI-52466 (GYKI), or both, relative to control (Cont). GYKI alone was not found to alter protein levels. GYKI was also unable to affect changes in the expression of all three protein species triggered by KA. All cultured data represent means±SEM from 3-4 independent experiments. $^{**}p<0.01$, $^{***}p<0.001$, ns = not statistically significant.

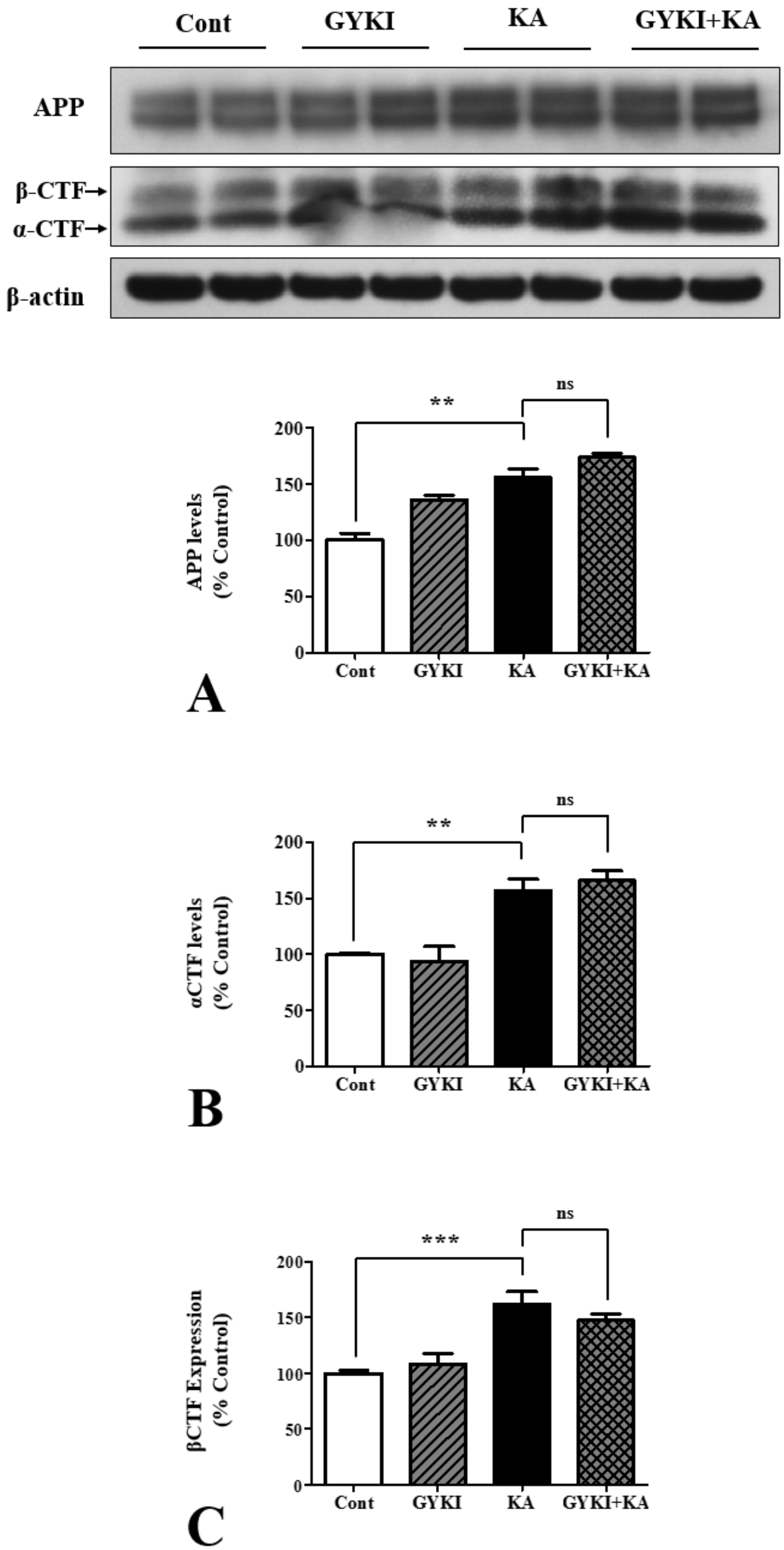

Cont
GYKI
KA
GYKI+KA
APP
β-CTF
α-CTF
β-actin
ns
**
APP levels (% Control)
200
150
100
50
0
Cont
GYKI
KA
GYKI+KA
A
ns
**
αCTF levels (% Control)
200
150
100
50
0
Cont
GYKI
KA
GYKI+KA
B
ns
***
βCTF Expression (% Control)
200
150
100
50
0
Cont
GYKI
KA
GYKI+KA
C

Fig. 3.14: A-C; Histograms showing the alteration in APP (A), α-CTF (B), and β-CTF (C) levels in rat primary astrocyte cells treated with 100μM KA over 3, 6, 12, 24, and 48h relative to control. In all cases, KA induced increases in protein expression that peaked around 12-24h post-treatment. All data represent means±SEM from 3-4 independent experiments. *$p<0.05$, **$p<0.01$.

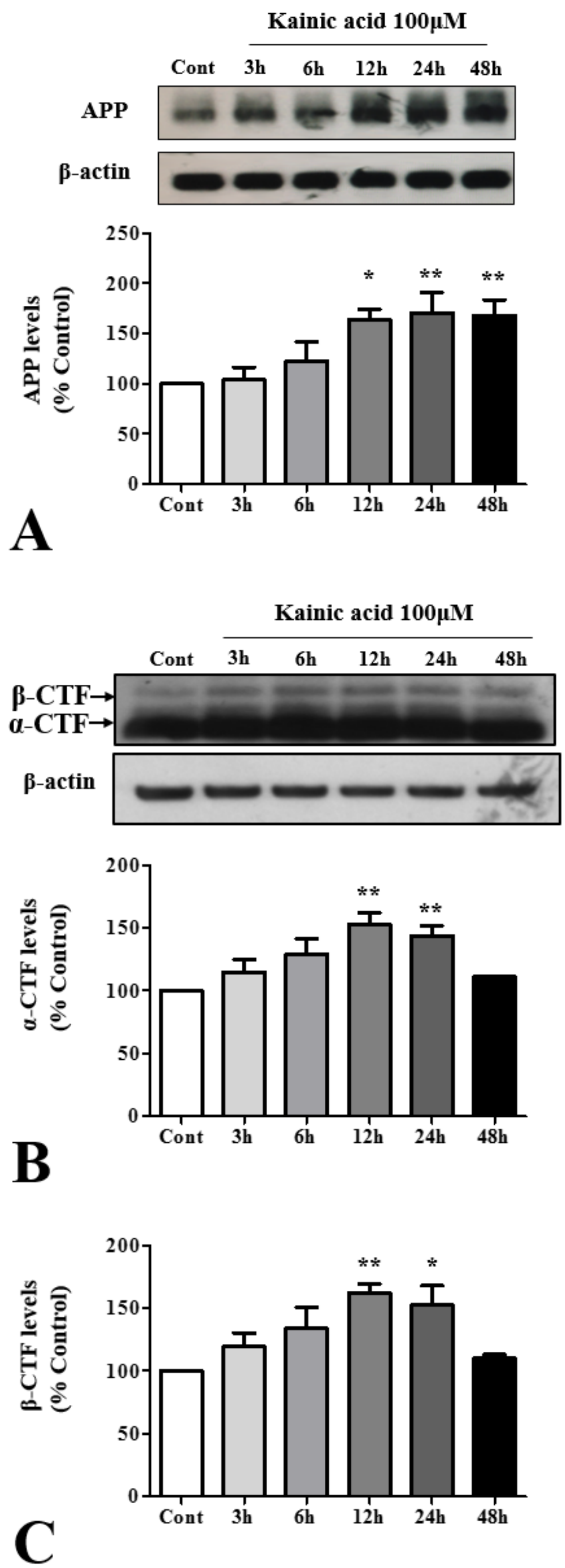
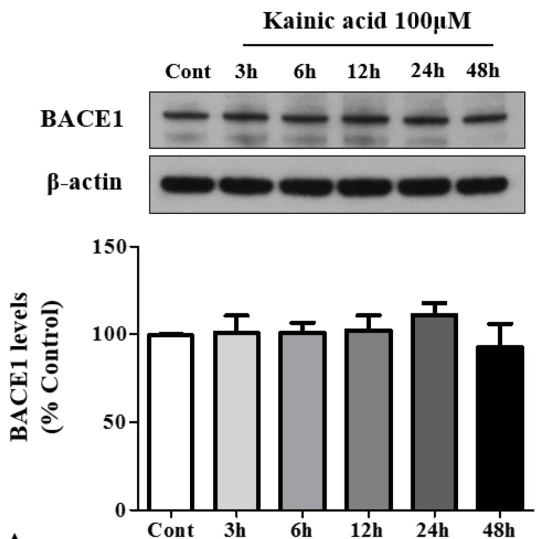
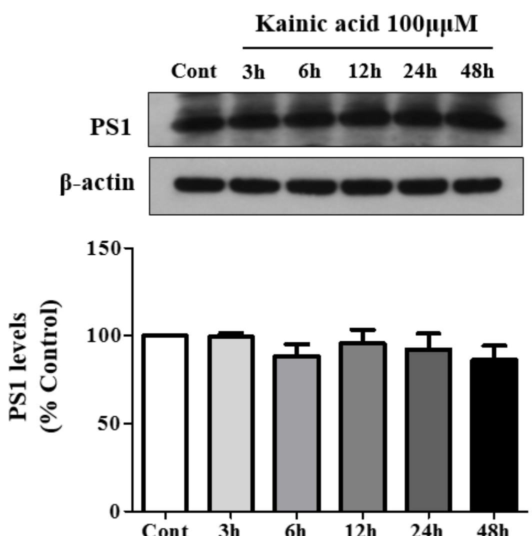

Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
APP
β-actin
APP levels (% Control)
0 50 100 150 200 250
*
**
**
Cont 3h 6h 12h 24h 48h
A
Kainic acid 100μM
Cont 3h 6h 12h 24h 48h
β-CTF
α-CTF
β-actin
α-CTF levels (% Control)
0 50 100 150 200
**
**
Cont 3h 6h 12h 24h 48h
B
β-CTF levels (% Control)
0 50 100 150 200
**
*
Cont 3h 6h 12h 24h 48h
C

Fig. 3.15: A and B; Histograms showing the alteration of BACE1 (A) and the catalytic PS1 subunit of γ-secretase (B) in rat primary astrocyte cells following 100μM of KA over time. KA did not affect the levels of these proteins at any time-point. All data represent means±SEM from 3-4 independent experiments.

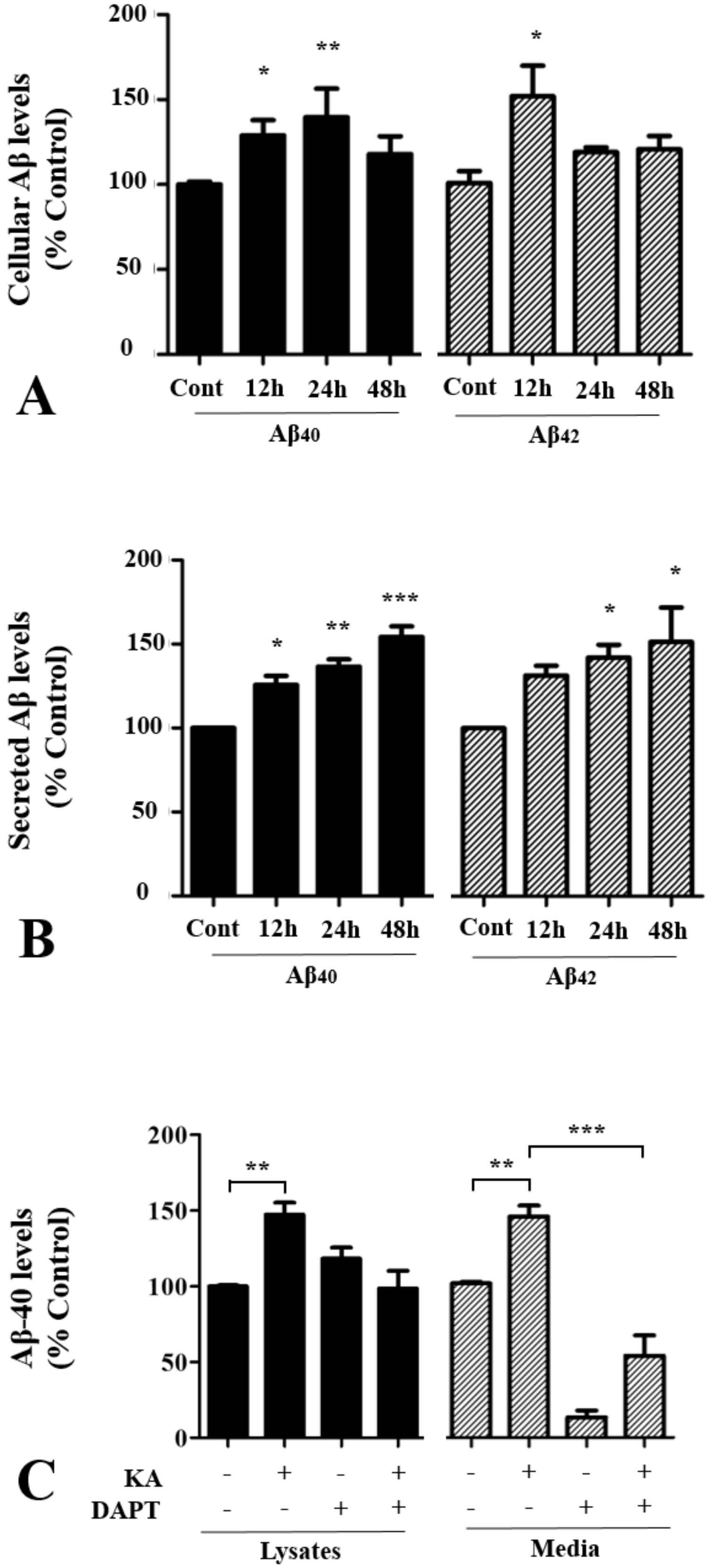

Kainic acid 100μM
Cont
3h
6h
12h
24h
48h
BACE1
β-actin
150
100
50
0
BACE1 levels
(% Control)
Cont
3h
6h
12h
24h
48h

A


Kainic acid 100μμM
Cont
3h
6h
12h
24h
48h
PS1
β-actin
150
100
50
0
PS1 levels
(% Control)
Cont
3h
6h
12h
24h
48h

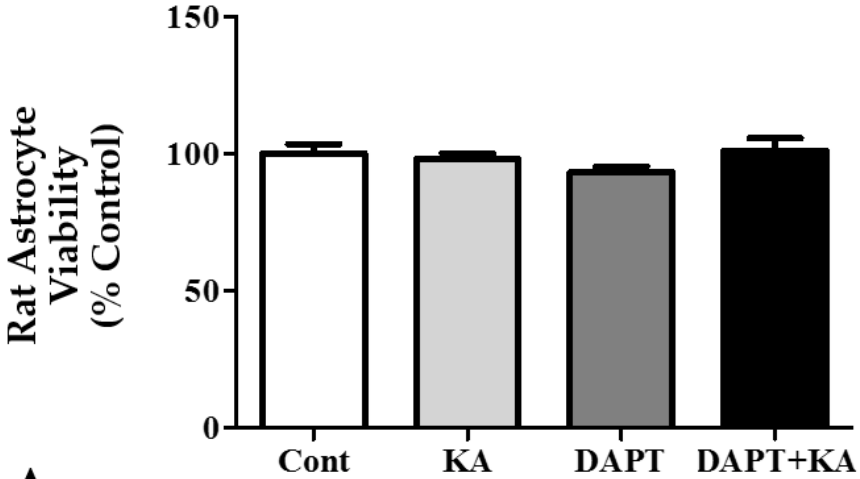
B

Fig. 3.16: A and B; Histograms showing the alteration in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in cell lysates (A) and media (B) of rat primary astrocytes treated with 100µM of KA over 12hr, 24hr and 48hr relative to control (Cont). Both $A\beta_{1-40}$ and $A\beta_{1-42}$ show a significant increase in the cell lysates as well as in the media following KA treatment. C; Histogram depicting the levels of $A\beta_{1-40}$ in primary rat astrocyte cultures treated with either KA, γ-secretase inhibitor DAPT, or a combination of the two (DAPT+KA) relative to control. Note that DAPT drastically reverses the increase in $A\beta_{1-40}$ levels triggered by KA and this effect is especially prominent in the conditioned media. All data represent means±SEM from 3-4 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

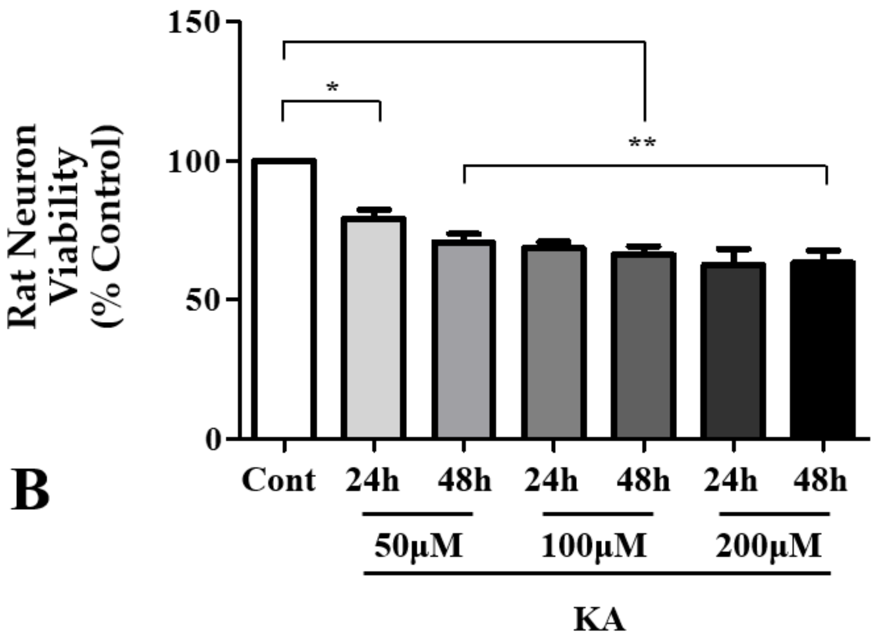


Cellular Aβ levels (% Control)
200
150
100
50
0
*
**
*
Cont 12h 24h 48h
Cont 12h 24h 48h
Aβ40
Aβ42
A
Secreted Aβ levels (% Control)
*
**
***
*
*
Cont 12h 24h 48h
Cont 12h 24h 48h
Aβ40
Aβ42
B
Aβ-40 levels (% Control)
**
**
***
KA
DAPT
Lysates
Media
C

Fig. 3.17: A. Histogram showing no change in the viability of cultured astrocytes following treatment with KA, DAPT or both, as measured by an MTT assay. B. Histogram showing the effect of KA on the viability of rat primary hippocampal cultured neurons as assessed by an MTT assay. KA was found to be toxic to neurons in relation to control. All data represent means±SEM from 3-4 independent experiments. *$p<0.05$, **$p<0.01$.

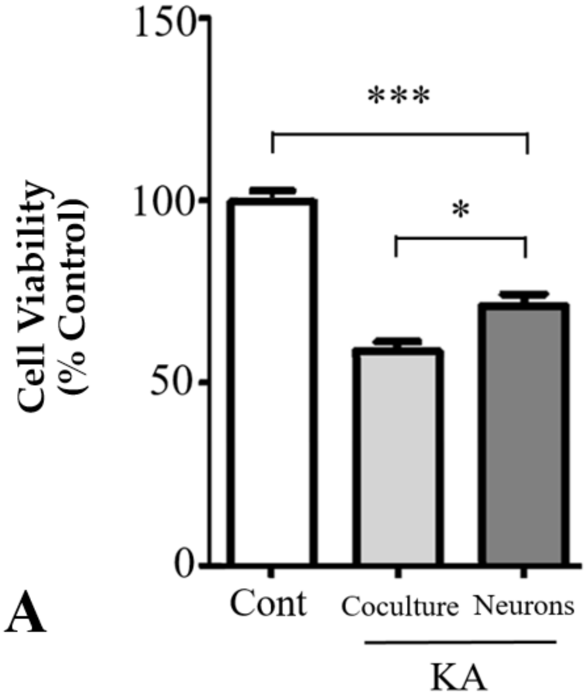

150
100
50
0
Rat Astrocyte Viability (% Control)
Cont
KA
DAPT
DAPT+KA

A

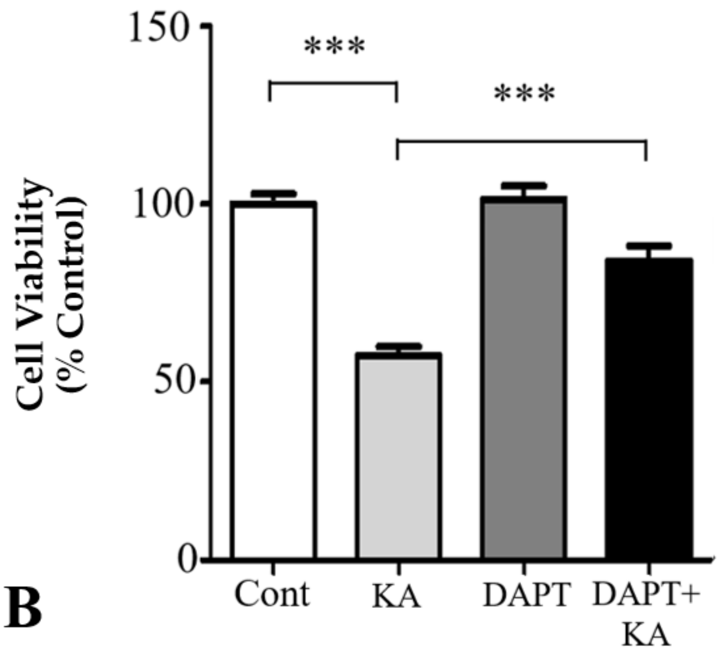

150
100
50
0
Rat Neuron Viability (% Control)
*
**
Cont
24h
48h
24h
48h
24h
48h
50µM
100µM
200µM
KA

B

Fig. 3.18: A. Histogram showing viability of KA-treated neuron/astrocyte co-cultures and pure neuronal cultures compared to control. A greater loss in cell viability was observed in co-culture conditions relative to pure neuronal cultures. B. Histogram showing the viability of neuron/astrocyte co-cultures in relation to control following treatment with KA, DAPT and DAPT+KA. KA-induced death of neurons was attenuated by DAPT pretreatment. All data represent means±SEM from 3-4 independent experiments. *p<0.05, ***p<0.001.


150
100
50
0
Cell Viability (% Control)
***
*
Cont
Coculture
Neurons
KA

A


150
100
50
0
Cell Viability (% Control)
***
***
Cont
KA
DAPT
DAPT+ KA

B

4. Discussion

The results of the present study suggest a hitherto unexplored mechanism of neuronal cell death involved in KA toxicity, centered on the enhanced production and secretion of Aβ peptides from activated astrocytes. This is supported by data which show that: i) KA triggers time- and dose-dependent increases in APP expression in human astrocytes; ii) this increase is accompanied by a significant enhancement of γ-secretase activity, and a corresponding upregulation of APP-cleavage products, including α-CTF, β-CTF, and $A\beta_{1\text{-}40/1\text{-}42}$ peptides; iii) these increases occur in absence of changes in the degradation/clearance rate of APP and its cleaved products; iv) the changes in APP/Aβ metabolism following KA treatment are mediated by the kainate receptor, as opposed to the glutamatergic NMDA or AMPA receptors; v) KA treatment of rat primary hippocampal astrocytes results in increased APP levels and enhanced Aβ production/secretion without compromising cell viability; vi) KA-induced degeneration of rat hippocampal cultured neurons is exacerbated by the presence of astrocytes, and vii) simultaneous application of a γ-secretase inhibitor DAPT was able to attenuate KA-mediated neuronal loss in mixed neuronal/astrocyte cultures. Altogether, these results indicate that activated astrocytes, by way of heightened APP expression and the increased production of Aβ peptides, play a critical role in KA-induced neurodegeneration. Given the neuropathological parallels between KA administration and human MTLE, it is possible that APP/Aβ peptides derived from astrocytes may also have a role in the degeneration of neurons in this epileptic condition.

Kainic acid-induced Aβ metabolism in astrocytes**:** Previous studies from our lab and others have reported that systemic KA administration can induce glial hypertrophy/proliferation along with the loss of neurons in the hippocampus of adult rats (Wang et al., 2005; Vincent and Mulle, 2009; Banerjee et al., 2015; Levesque et al., 2016). We also reported that the observed neurodegeneration triggered by the KA treatment coincided with a time-dependent elevation of APP and its processing enzymes in GFAP-expressing reactive astrocytes. The present study, using cultured human U373 astrocytoma and rat primary hippocampal astrocytes, further characterized the downstream implications of KA-triggered increases in APP production/processing.

Astrocytes are the most abundant glial cells in the central nervous system. They play vital roles in maintaining neuronal homeostasis by regulating trophic/metabolic support, neurotransmitter milieus, blood-brain barrier integrity, synaptic activity and synapse formation/remodeling (Barres, 2008; Belanger and Magistretti, 2009; Sidoryk-Wegrzynowicz et al., 2011; Nag 2011; Pekny et al., 2014). Normal astrocytes, unlike neurons, express very little APP and its processing enzymes, particularly BACE1 and the γ-secretase components (Beeson et al., 1994; Kodam et al., 2008; Siman and Salidas, 2004; Sun et al., 2002). Thus, neuronal cells appear to be the primary source for Aβ production in physiological conditions, whereas astrocytes fulfill a partial role in the clearance and degradation of the peptides (Osborn et al., 2016; Zhao et al., 2011). Upon activation, which may result from injury or diseases, astrocytes undergo specific modifications resulting in "reactive gliosis" – characterized by hypertrophy of cellular processes and upregulation of intermediate filament proteins including GFAP. Consequently, activated astrocytes lose some of their normal homeostatic functions and participate in inflammatory reactions that contribute to a variety of pathological changes (Belanger and Magistretti, 2009; Nag 2011; Sidoryk-Wegrzynowicz et al., 2011; Pekny et al., 2014; Batarseh et al., 2016). Concurrently, activated astrocytes have been shown to express APP, BACE1 and PS1 under various experimental conditions, such as cerebral ischemia, traumatic brain injury, excitotoxicity and cholesterol sequestration (Siman et al., 1989; Banati et al., 1995; Nihashi et al., 2001; Nadler et al., 2008; Kodam et al., 2010; Avila-Munoz and Arias, 2015). There is also evidence that activated astrocytes located in close proximity to Aβ-containing neuritic plaques in AD brains and in mutant APP transgenic mice exhibit higher levels of APP and/or its processing enzymes (Rossner et al., 2001; Hartlage-Rubsamen et al., 2003; Nagele et al., 2003; Simpson et al., 2010). Hence, it is possible that astrocyte activation under certain aberrant conditions may cause these cells to produce Aβ peptides which can contribute to pathological developments such as neuronal loss.

Earlier studies from our group and others have shown that KA treatment of adult rats results in a marked increase in the expression of APP and its processing enzymes in reactive astrocytes that coincides with the degeneration of hippocampal CA1-CA3 pyramidal neurons (Kodam et al., 2017) It is unclear, however, what the functional consequences of the elevated levels of APP and its processing enzymes are on the surrounding neurons, and whether this effect was mediated by direct activation of astrocytic kainate receptors or indirectly *via* other mechanisms. The present study using human glioblastoma U373 cells reveals that KA treatment enhances the levels of APP holoprotein in a time- and dose-dependent manner. This is accompanied by an increased level of α-/β-CTFs as well as intracellular/secretory levels of both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides. Interestingly, we did not observe any alteration in the steady state levels of the APP secretases; nevertheless, the activity of γ-secretase was found to be greatly increased in KA-treated U373 cells. The discrepancy between the steady state levels of γ-secretase complex and activity of this enzyme has been reported earlier (Ourdev et al., 2015) and is consistent with the evidence that four subunits of γ-secretase are tightly regulated by their stoichiometric interaction and ability to form stable complexes (Thinakaran et al., 1997; Takasugi et al., 2003). The observed changes in the APP holoprotein and its metabolites are evident in absence of any alterations in cell viability, suggesting that these changes are not due to the consequence of KA-induced toxicity. Additionally, the clearance/degradation of APP and its cleaved products are not altered following cycloheximide treatment, indicating that increased levels of CTFs and $A\beta_{1\text{-}40}$/$A\beta_{1\text{-}42}$ are likely the consequence of increased production as opposed to decreased clearance of the peptides. We also did not observe any marked alteration in two important Aβ-degrading enzymes, IDE and neprilysin. Interestingly, the effects of KA on APP metabolism in cultured astrocytes is inhibited by the kainate receptor antagonist ACET, but not by either the selective NMDA receptor antagonist AP5 or the selective AMPA receptor antagonist GYKI-52466, suggesting that the selective activation of kainate receptors especially underlies the increased levels and processing of APP in cultured astrocytes. Most of the results observed in human glioblastoma cells were also replicated in rat primary astrocytes, indicating that a similar mechanism may occur in both humans and rat models. Future experiments are needed to define the underlying mechanism by which KA treatment can lead to increased levels of the APP holoprotein in astrocytes. Nevertheless, given the fact that astrocytes vastly outnumber neurons in the brain (Kodam et al., 2017), our results show that activated astrocytes may serve as a potentially significant source of Aβ under certain pathological conditions.

The role of astrocyte-derived Aβ peptides in kainate-triggered neural death: KA is known to be neurotoxic, both in animal models and neuronal cell cultures (Levesque and Avoli, 2013). In agreement with prior studies (Ben-Ari and Cossart, 2000, Kodam et al., 2017), we report that treating rat primary neuronal cells with KA kills neurons in a manner proportional to the duration of treatment and the dose administered. Given that we observe increases in the amyloidogenic processing of APP and Aβ production/secretion in reactive astrocytes responding to KA, it is likely that these astrocyte-derived peptides may facilitate or compound KA-driven neuronal loss, especially considering previous studies which have shown rat Aβ peptides to be neurotoxic (Boyd-Kimball et al., 2004). In fact, we did observe that KA-induced cell loss is exacerbated in primary neuron/astrocyte co-cultures relative to primary neuron cultures. This additional cell death can be reversed by the application of the γ-secretase inhibitor DAPT, which ablates Aβ production and secretion from astrocytes; hence, these results collectively suggest that increased levels of Aβ peptides derived from astrocytes may directly be involved in the toxicity induced by KA.

Nevertheless, the precise manner by which Aβ facilitates neuronal cell death remains unclear, as Aβ peptides can induce cellular toxicity by a variety of mechanisms; accordingly, apoptotic, necrotic and autophagic markers are all found to be altered in neurons affected by KA (Wang et al., 2008; Wang and Qin, 2010). In the context of KA administration, it is possible that Aβ facilitates neuronal death by rendering cells more susceptible to excitotoxicity (Morimoto and Oda 2003). Studies have shown that Aβ can do this by directly interacting with NMDA receptors, thereby affecting cellular response to glutamatergic signaling. Alternatively, this peptide can positively modulate extracellular glutamate environment by potentiating glutamate release and inhibiting its reuptake, thereby indirectly affecting neuronal viability (Fernandez-Tome et al., 2004; Minkeviciene et al., 2009; Kabogo et al., 2010; Sanz-Blasco et al., 2016). Beyond excitotoxicity, Aβ peptides have been implicated in a number of neurotoxic processes including mitochondrial dysfunction, membrane permeabilization, ROS and oxidative stress, altered calcium homeostasis, and aberrant intracellular signaling which may also affect the survival of neurons (Carrillo-Mora et al., 2014).

The implication of astrocyte-derived Aβ peptides in mTLE: Recent literature identifies a tentative connection between Aβ peptides and MTLE. Some MTLE patients, for example, have been shown exhibit extracellular Aβ-containing plaques and increased levels of APP (Mackenzie and Miller, 1994; Sheng et al., 1994; Gouras et al., 1997; Sima et al., 2014); conversely, individuals afflicted with genetic overexpression of APP and/or Aβ, such as those who suffer from Down's syndrome (Trisomy 21) and some genetic early-onset forms of AD, are found to suffer from temporal and other forms of epilepsy at much higher frequencies than age-matched populations (Amatniek et al., 2006; Larner, 2010; Born, 2015, Chin and Scharfman, 2013). Nevertheless, our understanding of what contributions astrocyte-derived Aβ peptides have towards the development of MTLE pathology remains limited.

As mentioned previously, astrocytes normally do not express APP or secrete Aβ, instead playing a partial role in these peptides' clearance in addition to fulfilling functions necessary for proper neural homeostasis. As a result of the aberrant glutamatergic neurotransmission which drives seizures, astrocytes in MTLE are known to adopt a pathological reactive state which contributes to the sclerotic hippocampal syndrome (Tian et al, 2005; Blumcke et al., 2002). Our studies show that KA causes astrocytes to adopt a reactive phenotype that actively produces neurotoxic Aβ peptides; importantly, we demonstrate that this mechanism is mediated by the kainate receptor, as it can be attenuated by the application of a selective antagonist. Hence, it is highly likely that the glutamatergic currents which drive MTLE can likewise trigger astrocytes to adopt a reactive state that favors Aβ production and secretion. Once produced, astrocyte-derived Aβ peptides may play a role in facilitating the neurodegeneration associated with this condition through the various aforementioned mechanisms. Additionally, given that the kainate receptor is particularly concentrated in the CA1 and CA3 regions of the hippocampus (Vincent and Mulle, 2009), the implication of the kainate receptor in this process may shed some light as to why this particular structure is so adversely affected in MTLE.

In addition to inducing neuronal loss, reactive astrocytes and the Aβ peptides they produce may also contribute to the disease state of MTLE by promoting epileptogenesis. By adopting a reactive state, astrocytes lose their ability to regulate glutamate homeostasis, which can simultaneously exacerbate both neurotoxicity and promote the occurrence of seizures in the diseased hippocampus. Furthermore, Aβ peptides have been shown to potentiate the release (Kabogo et al., 2010; Revett et al., 2013; Sanz-Blasco et al., 2016) and inhibit the uptake of glutamate by astrocytes (Harris et al., 1996; Fernandez-Tome et al., 2004). Hence, it is likely that astrocyte-derived Aβ peptides themselves have an epileptogenic effect. Aβ is also known to trigger synapse atrophy and remodeling, and this likely compounds with the loss of neurons in MTLE to create excitatory network-level changes in the hippocampus; this may form a feed-forward loop promoting further neurodegeneration and epileptogenesis, thereby exacerbating the disease state

(Noebels, 2011; Palop and Mucke, 2010). This is supported by the evidence that: i) Aβ peptides can directly induce neuronal hyperexcitability and trigger epilepsy (Minkeviciene et al., 2009); ii) transgenic mice overexpressing Aβ peptide exhibit spontaneous seizures more frequently, and are more sensitive to induced seizures than wild-type mice (Del Vecchio et al., 2004; Palop et al., 2007; Westmark et al., 2008); iii) the prevalence of seizures is higher in AD cases than in control populations (Amatniek et al., 2006; Larner, 2010; Scharfman, 2012b; Born, 2015), and iv) immunization of mutant APP transgenic mice with Aβ peptide protects them from seizures (Mohajeri et al., 2002). Our results add to these findings by showing that KA-induced neuronal death is worsened in the presence of astrocytes and that, notably, this effect is mitigated by inhibiting Aβ synthesis by chemically blocking the activity of γ-secretase. Collectively, these results not only highlight the significance of astrocytic Aβ in MTLE pathology, but also raise the possibility that lowering Aβ levels may attenuate seizure generation and neuronal loss.

Future directions: The results of this project offer plenty of avenues for future work. The next step in this line of research would be to determine by which molecular mechanisms Aβ induces widespread neuronal death in the KA model. One particularly interesting subject that is worth looking into is astrocyte-derived Aβ and its effect on aberrant tau phosphorylation and pathology. Tau is a predominantly neuronal protein that serves to stabilize microtubule filaments. Under pathological conditions, this protein becomes hyperphosphorylated and forms intracellular aggregates known as neurofibrillary tangles (NFT). NFTs, much like amyloid plaques composed of Aβ aggregates, are a primary pathology of AD; similarly, they are widely neurotoxic and form the crux of various neurodegenerative diseases known collectively as tauopathies. Recently, tau dysfunctions have garnered much attention in the context of MTLE, as studies have shown putative development of NFTs in brain samples collected from hippocampal biopsies of 31 MTLE patients (Tai et al., 2016). Notably, the NFT development in these patients were comparable to Braak patterns in AD. Furthermore, glutamate antagonists have been found to protect cells against Aβ toxicity by reducing the activity of kinase enzymes responsible for phosphorylating tau (Tremblay et al., 2000; Song et al., 2008). Meanwhile, *in vivo* treatments with NMDA or KA have been shown to reduce tau dephosphorylation by diminishing the activity of its protein phosphatases, thereby demonstrating that excessive glutamatergic activity can enable a pathological hyperphosphorylated state. Conversely, tau appears to propagate the deleterious effects of these glutamate receptor agonists, as genetically reducing tau in mouse models has been shown to effectively attenuate the effect of epileptogenic agents (Tremblay et al., 2000). In separate studies, reducing tau was shown to prevent synaptic impairments and hippocampal remodeling, suppress seizure activity, and mitigate Aβ-induced cognitive impairments (Brunden, 2009, Roberson et al., 2011). Considering the close interrelationship which exists between Aβ and tau hyperphosphorylation, to the point where some researchers have characterized Aβ as the "trigger" and tau the "bullet" of AD, it is highly likely that Aβ may potentiate neuronal death by affecting tau phosphorylation. This concept has certainly not been explored with regards to KA-treated astrocytes, and hence makes a logical next step in the investigation related to this project.

Another consideration worth investigating is the effect of astrocyte-derived Aβ on epileptogenesis in KA-treated animals. Given the deleterious effect of Aβ on glutamate signaling, synapse function, and cell death outlined above, it is likely that these peptides may be responsible for propagating seizures in addition to contributing to the neurodegeneration observed in KA toxicity. Furthermore, it has been shown that treatment with glutamate receptor antagonists such as memantine and antiepileptic drugs such as levetiracetam can partially reverse AD-related pathology (Bakker et al., 2012; Sanchez et al., 2012; Zhang et al., 2014; Xiao, 2016). Hence, it would be worthwhile to explore this concept by specifically targeting reactive APP-expressing astrocytes and assessing whether this can alleviate KA pathology; these studies may provide the groundwork for new, valuable treatment strategies for patients with MTLE which targeting astrocyte activation and/or production of Aβ.

References

Acharya JN and Acharya VJ. 2014. Epilepsy in the elderly: Special considerations and challenges. *Ann Indian Acad Neurol,* 17(1): S18–S26.

Acosta I, Vale F, Tatum WO 4th, and Benbadis SR. 2008. Epilepsy surgery after age 60. *Epilepsy Behav,* 12(2):324–325.

Algreeshah, F. (2014). *Psychiatric Disorders Associated With Epilepsy*. [online] Retrieved from: http://emedicine.medscape.com/article/1186336-overview [Accessed: 26 Mar 2014].

Amatniek JC, Hauser WA, Del Castillo-Castaneda C, Jacobs DM, Marder K, Bell K, et al. 2006. Incidence and predictors of seizures in patients with Alzheimer's disease. *Epilepsia,* 47:867-72.

Anderson CM, and Swanson RA. 2000. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia*, 32(1):1-14.

Al-Otaibi F, Baeesa SS, Parrent AG, Girvin JP, and Steven D. 2012. Surgical techniques for the treatment of temporal lobe epilepsy. *Epilepsy Res Treat*, 2012:374848.

Avila-Muñoz E, and Arias C. 2015. Cholesterol-induced astrocyte activation is associated with increased amyloid precursor protein expression and processing. *Glia,* 63:2010-22.

Bakker A, Krauss GL, Albert MS, Speck CL, Jones LR, Stark CE, et al. 2012. Reduction of hippocampal hyperactivity improves cognition in amnestic mild cognitive impairment. *Neuron,* 74:467-74.

Banati RB, Gehrmann J, Wiessner C, Hossmann KA, and Kreutzberg GW. 1995. Glial expression of the β-amyloid precursor protein (APP) in global ischemia. *J Cereb Blood Flow Metab,* 15:647-54.

Bancaud J, Brunet-Bourgin F, Chauvel P, and Halgren E. 1994. Anatomical origin of deja vu and vivid 'memories' in human temporal lobe epilepsy. *Brain,* 117(1):71-90.

Banerjee M, Sasse A, Wang Y, Maulik M, and Kar S. 2015. Increased levels and activity of cathepsins B and D in kainate-induced toxicity. *J Neurosci,* 284:360-73.

Baranello RJ, Bharani KL, Padmaraju V, Chopra N, Lahiri DK, Greig NH, Pappolla MA, et. al. 2015. Amyloid-beta protein clearance and degradation (ABCD) pathways and their role in Alzheimer's disease. *Curr Alzheimer Res,* 12(1):32–46.

Barres BA. 2008. The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron,* 60(3):430-40.

Batarseh YS, Duong QV, Mousa YM, Al Rihani SB, Elfakhri K, Kaddoumi A. 2016. Amyloid-β and astrocytes interplay in amyloid-β related disorders. *Int J Mol Sci*, 17(3):338

Beeson JG, Shelton ER, Chan HW, and Gage FH. 1994. Differential distribution of amyloid protein precursor immunoreactivity in the rat brain studied by using five different antibodies. *J Comp Neurol,* 342:78-96.

Belanger M, and Magistretti PJ. 2009. The role of astroglia in neuroprotection. *Dial Clin Neurosci,* 11(3):281-95.

Ben-Ari Y and Cossart R. 2000. Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci*, 23:580-587.

Berliocchi L, Bano D, and Nicotera P. 2005. Ca^{2+} signals and death programmes in neurons. *Phil Trans R Soc B Bioll Sci,* 360:2255-58.

Blümcke I, Thom M, and Wiestler OD. 2002. Ammon's horn sclerosis: a maldevelopmental disorder associated with temporal lobe epilepsy. *Brain Pat,* 12(2):199-211.

Bordji K, Becerril-Ortega J, Nicole O, and Buisson A. 2010. Activation of extrasynaptic, but not synaptic, NMDA receptors modifies amyloid precursor protein expression pattern and increases amyloid-β production*. J. Neurosci,* 30:15927-15942.

Born HA. 2015. Seizures in Alzheimer's disease. *J Neurosci,* 286:251-63.

Boyd-Kimball D, Sultana R, Mohmmad-Abdul H, Butterfield DA. 2004. Rodent Aβ (1-42) exhibits oxidative stress properties similar to those of human Aβ (1-42): Implications for proposed mechanisms of toxicity. *J Alzheimers Dis,* 6:515-25.

Braak H, and Braak E. 1991. Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol,* 82(4):239-59.

Brunden KR, Trojanowski JQ, and Lee VM. 2009. Advances in tau-focused drug discovery for Alzheimer's disease and related tauopathies. *Nat Rev Drug Disc*, 8(10):783-93.

Caccamo A, Oddo S, Sugarman MC, Akbari Y, and Laferla FM. 2005. Age- and region-dependent alterations in Abeta-degrading enzymes: implications for Abeta-induced disorders. *Neurobiol Aging,* 5(26):645-54.

Campbell SL and Hablitz JJ. 2004. Glutamate transporters regulate excitability in local networks in rat neocortex. *Neurosci,* 127:625–35.

Carrillo-Mora P, Luna R, and Colin-Barenque L. 2014. Amyloid beta: multiple mechanisms of toxicity and only some protective effects? *Oxid Med Cell Longev,* 2014:795375.

Cavus I, Kasoff WS, Cassaday MP, Jacob R, Gueorguieva R, Sherwin RS, et al. 2005. Extracellular metabolites in the cortex and hippocampus of epileptic patients. *Ann Neurol,* 57:226–35.

Chávez-Gutiérrez L, Bammens L, Benilova I, Vandersteen A, Benurwar M, Borgers M, Lismont S, et al. 2012. The mechanism of γ-Secretase dysfunction in familial Alzheimer disease. *EMBO J,* 31(10):2261–74.

Chesneau V, Vekrellis K, Rosner MR, and Selkoe DJ. 2000. Purified recombinant insulin-degrading enzyme degrades amyloid *β*-protein but does not promote its oligomerization. *J Biochem,* 351:509-16.

Chin J and Scharfman HE. 2013. Shared cognitive and behavioral impairments in epilepsy and Alzheimer's disease and potential underlying mechanisms. *Epilepsy Behav,* 26(3):343-51.

Choi DW, Koh JY, and Peters S. 1988. Pharmacology of glutamate toxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci,* 8(1):185-96

Choi DW. Excitotoxic cell death. 1992. *J Neurobiol,* 23(9):1261-76

Clippingdale AB, Wade JD, and Barrow CJ. 2001. The amyloid-β peptide and its role in Alzheimer's disease. *J Pep Sci,* 7(5):227-249

Cole SL, and Vassar R. 2008. The role of amyloid precursor protein processing by BACE1, the beta-secretase, in Alzheimer disease pathophysiology. *J Biol Chem*, 283(44):29621-5.

Dargan SL, Clarke VR, Alushin GM, Sherwood JL, Nistico R, Bortolotto ZA, Ogden AM, et al. 2009. ACET is a highly potenta and specific kainate receptor antagonist: characterization and effects on hippocampal mossy fibre function. *Neuropharmacol* 56(1):121-30.

Del Vecchio RA, Gold LH, Novick SJ, Wong G, and Hyde LA. 2004. Increased seizure threshold and severity in young transgenic CRND8 mice. *Neurosci. Letts*, 367:164-67.

Dong X, Wang Y, and Qin Z. 2009. Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Act Pharm Sin,* 30:379-87.

During MJ and Spencer DD. 1993. Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. *Lancet*, 341:1607–10.

Eid T, Williamson A, Lee TSW, Petroff OA, and de Lanerolle NC. 2008. Glutamate and astrocytes - key players in human mesial temporal lobe epilepsy? *Epilepsia,* 49(2):42-52.

Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, et al. 2004. Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. *Lancet,* 363:28–37.

Fernandez-Tome P, Brera B, Arevalo M, and de Ceballos ML. 2004. β-amyloid25-35 inhibits glutamate uptake in cultured neurons and astrocytes: modulation of uptake as a survival mechanism. *Neurobiol Dis,*15:580-89.

Glick D, Barth S, and Macleod KF. 2010. Autophagy: cellular and molecular mechanisms. *J Pathol,* 221(1):3–12.

Gouras GK, Relkin NR, Sweeney D, Munoz DG, Mackenzie IR, and Gandy S. 1997. Increased apolipoprotein E epsilon 4 in epilepsy with senile plaques. *Ann Neurol,* 41:402-4.

Haass C, Kaether C, Thinakaran G, and Sisodia S. 2012. Trafficking and proteolytic processing of APP. *Cold Spring Harb Perspect* Med, 2(5):a006270.

Harris ME, Wang Y, Pedigo NW, Hensley K, Butterfield DA, and Carney JM. 1996. Amyloid β peptide (25-35) inhibits Na^+-dependent glutamate uptake in rat hippocampal astrocyte cultures. *J Neurochem*, 67:277-86.

Hartlage-Rubsamen M, Zeitschel U, Apelt J, Gartner U, Franke H, Stahl T, et al. 2003. Astrocytic expression of the Alzheimer's disease β-secretase (BACE1) is stimulus-dependent. *Glia,* 41:169-79.

Hauser WA, Annegers JF, and Rocca WA. 1996. Descriptive epidemiology of epilepsy: contributions of population-based studies from Rochester, Minnesota. *Mayo Clin Proc*, 71:576–86.

Hazra A, Gu F, Aulakh A, Berridge C, Eriksen JL, and Vziburkus J. 2013. Inhibitory neuron and hippocampal circuit dysfunction in an aged mouse model of Alzheimer's disease. *Plos One*, 8(5):64318.

Hirtz D, Thurman D, Gwinn-Hardy K, Mohamed M, Chaudhuri A, and Zalutsky R. 2007. How common are the "common" neurologic disorders? *Neuro,* 68(5):326-37

Huang SM, Mouri A, Kokubo H, Nakajima R, Suemoto T, Higuchi M, et al. 2006. Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. *J Biol Chem,* 281(26):17941–51.

Johnstone M, Gearing AJ, and Miller KM. 1999. A central role for astrocytes in the inflammatory response to β-amyloid; chemokines, cytokines and reactive oxygen species are produced. *J Neuroim,* 93(1-2):182-93.

Kabogo, D., G. Rauw, A. Amritraj, G. Baker, and S. Kar. 2010. β-amyloid-related peptides potentiate K^+-evoked glutamate release from adult rat hippocampal slices. *Neurobiol Aging*, 31:1164-1172.

Kaplan PW, Fisher RS, Jobst B, and Williamson P. 2005. Typical Seizures Originating in the Temporal Lobes. *Demos Medical Publishing*.

Kew JN and Kemp JA. 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmaco*, 179(1):4-29.

Kodam A, Vetrivel KS, Thinakaran G, and Kar S. 2008. Cellular distribution of gamma-secretase subunit nicastrin in the developing and adult rat brains. *Neurobiol Aging,* 29:724-38.

Kodam A, Maulik M, Peake K, Amritraj A, Vetrivel K, Thinakaran G, et al. 2010. Altered levels and distribution of APP and its processing enzymes in Niemann-Pick Type C1-deficient mouse brains. *Glia,* 58:1267-81.

Kodam A, Ourdev D, Maulik M, Hariharakrishnan J, Banerjee M, Wang Y, Kar S. 2017. A role for astrocyte-derived amyloid β peptides in the degeneration of neurons in an animal model of Temporal Lobe Epilepsy. UNPUBLISHED MANUSCRIPT.

Lalonde R, Fukuchi K, and Strazielle C. 2012. Neurologic and motor dysfunctions in APP transgenic mice. *Rev Neurosci*, 23:363-79.

Larner AJ. 2010. Epileptic seizures in AD patients. *Neuromol Med,* 12:71-7.

Leem JY, Saura CA, Pietrzik C, Christianson J, Wanamaker C, King LT, Veselits ML, et al. 2002. A role for presenilin 1 in regulating the delivery of amyloid precursor protein to the cell surface. *Neurobiol Dis,* 11(1):64-82.

Leissring MA, Farris W, Chang AY, Walsh DM, Wu X, Sun X, Frosch MP and Selkoe DJ. 2003. Enhanced proteolysis of β-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron,* 40:1087-93.

Lesné S, Docagne F, Gabriel C, Liot G, Lahiri DK, Buée L, Plawinski L, et al. 2003. Transforming growth factor-beta 1 potentiates amyloid-beta generation in astrocytes and in transgenic mice. *J Biol Chem,* 278(20):18408-18.

Lévesque M and Avoli M. 2013. The kainic acid model of temporal lobe epilepsy *Neurosci Biobehav Rev,* 37:2887–99

Lévesque M, Avoli M, and Bernard C. 2016. Animal models of temporal lobe epilepsy following systemic chemoconvulsant administration. *J Neurosci Methods*, 260:45-52.

Mackenzie IR and Miller LA. 1994. Senile plaques in temporal lobe epilepsy. *Acta Neuropath,* 87 (5):504-510

Marr RA, Guan H, Rockenstein E, Kindy M, Gage FH, et al. 2004. Neprilysin regulates amyloid beta peptide levels. *J Mol Neuro,* 22(1-2):5-11.

Maulik M, Peake K, Chung J, Wang Y, Vance JE, and Kar S. 2015. APP overexpression in absence of NPC1 excerbates metabolism of amyloidogenic proteins of Alzheimer's disease. *Hum Mol Genetics,* 24:7132-50.

McKhann GM 2nd, Wenzel HJ, Robbins CA, Sosunov AA, and Schwartzkroin PA. 2003. Mouse strain differences in kainic acid sensitivity, seizure behavior, mortality, and hippocampal pathology. *Neurosci,* 122(2):551-61.

McLachlan RS, Chovaz CJ, Blume WT, and Girvin JP. 1992. Temporal lobectomy for intractable epilepsy in patients over age 45 years. *Neurology,* 42(3 Pt 1):662–5

Meilandt WJ, Cisse M, Ho K, Wu T, Esposito LA, Scearce-Levie K, Cheng IH, Yu GO, and Mucke L. 2009. Neprilysin overexpression inhibits plaque formation but fails to reduce pathogenic Aβ oligomers and associated cognitive deficits in human amyloid precursor protein transgenic mice. *J Neurosci,* 29(7):1977–86.

Minkeviciene R, Rheims S, Bobszay MB, Zilberter M, Hartikainen J, Fulop L, et al. 2009. Amyloid β-induced neuronal hyperexcitability triggers progressive epilepsy. *J Neurosci,* 29:3453-62.

Mohajeri MH, Saini K, Schultz JG, Wollmer MA, Hock C, and Nitsch RM. 2002. Passive immunization against β-amyloid peptide protects central nervous system (CNS) neurons from increased vulnerability associated with an Alzheimer's disease-causing mutation. *J Biol Chem*, 277:33012-17.

Morimoto K and Oda T. 2003. Kainate exacerbates β-amyloid toxicity in rat hippocampus. *Neurosci. Letts*, 340:242-4.

Nadler Y, Alexandrovich A, Grigoriadis N, Hartmann T, Rao KS, Shohami E, and Stein R. 2008. Increased expression of the gamma-secretase components presenilin-1 and nicastrin in activated astrocytes and microglia following traumatic brain injury. *Glia,* 56:552-67.

Nag S. 2011. Morphology and properties of astrocytes. *Methods Mol Biol,* 686:69-100

Nagele RG, D'Andrea MR, Lee H, Venkataraman V, Wang HY. 2003. Astrocytes accumulate Aβ42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains. *Brain Res,* 971:197-209.

Nihashi T, Inao S, Kajita Y, Kawai T, Sugimoto T, Niwa M, et al. 2001. Expression and distribution of beta amyloid precursor protein and beta amyloid peptide in reactive astrocytes after transient middle cerebral artery occlusion. *Acta Neurochir*, 143:287-95.

Nixon RA. 2007. Autophagy, amyloidogenesis and Alzheimer disease. *J Cell Sci,* 120(23):4081-91.

Noebels J. 2011. A perfect storm: converging paths of epilepsy and Alzheimer's dementia intersect in the hippocampal formation. *Epilepsia*, 52(s1):39-46.

O'Brien RJ and Wong PC. 2011. Amyloid precursor protein processing and Alzheimer's disease. *Ann Rev Neurosci,* 34:185

Olney J, Collins R, and Sloviter R. 1985. Excitotoxic mechanisms of epileptic brain damage. *Adv Neurol*, 44:857-77.

Osborn LM, Kamphuis W, Wadman WJ, and Hol EM. 2016. Astrogliosis: an integral player in the pathogenesis of Alzheimer's disease. *Prog Neurobiol,* 144:121-41

Ourdev D, Foroutanpay BV, Wang Y, Kar S. 2015. The effect of Aβ1-42 oligomers on APP processing and Aβ1-40 generation in cultured U-373 astrocytes. *Neurodegener Dis*, 15(6):361-8.

Oyegbile T, Dow C, Jones J, Bell B, Rutecki P, Sheth R, Seidenberg M, and Hermann B. 2004. The nature and course of neuropsychological morbidity in chronic temporal lobe epilepsy. *Neurol,* 62(10):1736-42.

Palop JJ, Chin J, and Mucke L. 2006. A network dysfunction perspective on neurodegenerative diseases. *Nature,* 443(7113):768-73.

Palop JJ, Chin J, Roberson ED, Wang J, Thwin MT, Bien-Ly N, Yoo J, Ho KO, Yu GQ, Kreitzer A, Finkbeiner S, Noebels JL, and Mucke L. 2007. Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. *Neuron,* 55:697-711.

Palop JJ and Mucke L. 2010. Amyloid-β induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nat Neurosci,* 13(7):812–18.

Paternain AV, Morales M, Lerma Juan. 1995. Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron,* 14:185-9.

Pekny M and Pekna M. 2014. Astrocyte reactivity and astrogliosis: costs and benefits. *Physiol Rev,* 94(4):1077-98.

Poirier R, Wolfer DP, Welzl H, Tracy J, Galsworthy MJ, Nitsch RM and Mohajeri MH. 2006. Neuronal neprilysin overexpression is associated with attenuation of Aβ-related spatial memory deficit. *Neurobiol Dis,* 24:475–83.

Powell EM. 2014. Shedding the epilepsy comorbidity in Alzheimer's disease. *Epilepsy Curr,* 14(4): 211–12.

Proper EA, Hoogland G, Kappen SM, Jansen GH, Rensen MG, Schrama LH, van Veelen CW, et al. 2002. Distribution of glutamate transporters in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy. *Brain,* 125(1):32-43.

Revett TJ, Baker GB, Jhamandas J and Kar S. 2013. Glutamate system, amyloid beta peptides and tau protein: functional interrelationships and relevance to Alzheimer disease pathology. *J Psy Neurosci,* 38(1):6.

Roberson ED, Halabisky B, Yoo JW, Yao J, Chin J, Yan F, Wu T, et al. 2011. Amyloid-β/Fyn-induced synaptic, network, and cognitive impairments depend on tau levels in multiple mouse models of Alzheimer's disease. *J Neurosci,* 31(2):700-11.

Rossner S, Apelt J, Schliebs R, Perez-Polo JR, and Bigl V. 2001. Neuronal and glial β-secretase (BACE) protein expression in transgenic Tg2576 mice with amyloid plaque pathology. *J Neurosci Res,* 64:437-46.

Rush T and Buisson A. 2014. Reciprocal disruption of neuronal signaling and Aβ production mediated by extrasynaptic NMDA receptors: a downward spiral. *Cell Tissue Res*, 356:279-86.

Saido T and Leissring MA. 2012. Proteolytic degradation of amyloid β-protein. *Cold Spring Harb Perspect Med,* 2(6):a006379.

Sanchez PE, Zhu L, Verret L, Vossel KA, Orr AG, Cirrito JR, et al. 2012. Levetiracetam suppresses neuronal network dysfunction and reverses synaptic and cognitive deficits in an Alzheimer's disease model. *Proc Natl Acad Sci*, 109(42):2895-903.

Sanz-Blasco S, Piña-Crespo JC, Zhang X, McKercher SR, Lipton SA. 2016. Levetiracetam inhibits oligomeric Aβ-induced glutamate release from human astrocytes. *Neuroreport,* 27:705-9.

Sattler R and Tymianski M. 2001. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol Neurobiol,* 24(1-3):107-29.

Scarmeas N, Honig LS, Choi H, Cantero J, Br TJ, Blacker D, Albert M, Amatniek JC, Marder K, Bell K, et al. 2009. Seizures in Alzheimer disease: who, when, and how common? *Arch Neurol,* 66(8):992-7

Scharfman HE. 2007. The Neurobiology of Epilepsy. *Curr Neurol Neurosci Rep,* 7(4): 348–54.

Scharfman HE. 2012a. "Untangling" Alzheimer's disease and epilepsy. *Epilepsy Curr*, 12:178-83.

Scharfman HE. 2012b. Alzheimer's disease and epilepsy: insight from animal models. *Future Neurol,* 7:177-92.

Seifert G, Carmignoto G, and Steinhauser C. 2010. Astrocyte dysfunction in epilepsy. *Brain Res, Rev*, 63:212-21.

Selkoe DJ. 2001. Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. *J Alzheimers Dis,* 3(1):75-80.

Sheng JG, Boop FA, Mrak RE, and Griffin WS. 1994. Increased neuronal beta-amyloid precursor protein expression in human temporal lobe epilepsy: association with interleukin-1 alpha immunoreactivity. *J Neurochem*, 63:1872-9.

Sidoryk-Wegrzynowicz M, Lee E, Mingwei N, Aschner M. 2011. Distruption of astrocytic glutamate turnover by manganese is mediated by the protein kinase C pathway. *Glia,* 59(11):1732-43.

Sima X, Xu J, Li J, Zhong W, and You C. 2014. Expression of β-amyloid precursor protein in refractory epilepsy. *Mol Med Rep,* 9:1242-48.

Siman R, and Salidas S. 2004. Gamma-secretase subunit composition and distribution in the presenilin wild-type and mutant mouse brain. *J Neurosci,* 129:615-28.

Siman R, Card JP, Nelson RB and Davis LG. 1989. Expression of β-amyloid precursor protein in reactive astrocytes following neuronal damage. *Neuron,* 3:275-285.

Simpson JE, Ince PG, Lace G, Forster G, Shaw PJ, Matthews F, et al. 2010. Astrocyte phenotype in relation to Alzheimer-type pathology in the aging brain. *Neurobiol Aging*, 31:578-90.

Song M, Rauw G, Baker G and Kar S. 2008. Memantine protects rat cortical cultured neurons against β-amyloid-induced toxicity by attenuating tau phosphorylation. *Euro J Neurosci,* 28 (10):1989-2002

Sun A, Koelsch G, Tang J, and Bing G. 2002. Localization of beta-secretase memapsin 2 in the brain of Alzheimer's patients and normal aged controls. *Exp Neurol*, 175:10-22.

Tai XY, Koepp M, Duncan JS, Fox N, Thompson P, Baxendale S, Liu JY, et al. 2016. Hyperphosphorylated tau in patients with refractory epilepsy correlates with cognitive decline: a study of temporal lobe resections. *Brain,* 139(9):2441-55.

Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, Thinakaran G, et al. 2003. The role of presenilin cofactors in the gamma-secretase complex. *Nature*, 422(6930):438-41.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, et. al. 1997. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science,* 276(5319):1699-702.

Tian GF, Azmi H, Takano T, Xu Q, Peng W, Lin J, Oberheim N, et al. 2005. An astrocytic basis of epilepsy. *Nat Med*, 11(9):973-81.

Téllez-Zenteno JF and Hernández-Ronquillo L. 2012. A Review of the Epidemiology of Temporal Lobe Epilepsy Epilepsy Research and Treatment. *Epilepsy Res Treat,* 2012:630853.

Thinakaran G, Harris CL, Ratovitski T, Davenport F, Slunt HH, Price DL, et al. 1997. Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *J Biol Chem,* 272:28415–22.

Tremblay R, Chakravarthy B, Hewitt K, Tauskela J, Morley P, Atkinson T, Durkin JP. 2000. Transient NMDA receptor inactivation provides long-term protection to cultured cortical neurons from a variety of death signals. *J Neurosci*, 20:7183-92.

Tung YT, Wang BJ, Hu MK, Hsu WM, Lee H, Huang WP, and Liao YF. 2012. Autophagy: a double-edged sword in Alzheimer's disease. *J Biosci,* 37(1):157-65.

Vincent P and Mulle C. 2009. Kainate receptors in epilepsy and excitotoxicity. *Neuroscience,* 158: 309-23.

Volknandt W, Küster F, Wilhelm A, Obermüller E, Steinmann A, Zhang L, and Zimmermann H. 2002. Expression and allocation of proteins of the exo-endocytotic machinery in U373 glioma cells: similarities to long-term cultured astrocytes. *Cell Mol Biol,* 22:153-69

Wang Y, Han R, Liang ZQ, Wu JC, Zhang XD, Gu ZL, Qin ZH. 2008. An autophagic mechanism is involved in apoptotic death of art striatal neurons induced by the non-N-methyl-D-aspartate receptor agonist kainic acid. *Autophagy*, 4:214-26.

Wang Y, and Qin ZH. 2010. Molecular and cellular mechanisms of excitotoxic neuronal death. *Apoptosis,* 15:1382-402.

Wang Y, V Buggia-Prevot V, Zavorka ME, Bleackley RC, MacDonald RG, Thinakaran G, Kar S. 2015. Overexpression of the insulin-like growth factor-II receptor increases β-amyloid production and affects cell viability. *Mol Cell Biol*, 35:2368-84.

Wang Q, Yu S, Simonyi A, Sun GY, and Sun A.Y. 2005. Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Mol Neurobiol*, 31:3-15.

Wei, Z., M.S. Song, D. MacTavish, J.H. Jhamandas, and S. Kar. 2008. Role of calpain and caspase in β-amyloid-induced cell death in rat primary septal cultured neurons. *Neuropharmacol,* 54:721-33.

Wieser H. 2004. ILAE Commission Report. Mesial temporal lobe epilepsy with hippocampal sclerosis. *Epilepsia,* 45 (6):695-714.

Westmark CJ, Westmark PR, Beard AM, Hildebrandt SM, and Malter JS. 2008. Seizure susceptibility and mortality in mice that overexpress amyloid precursor protein. *Int J Clin Exp Pathol*, 1:157-68.

Wolfe MS. 2010. Structure, Mechanism and Inhibition of γ-Secretase and Presenilin-Like Proteases. *Biol Chem,* 391(8):839–47.

Wragg RE and Jeste DV. 1989. Overview of Depression and Psychosis in Alzheimer's Disease. *Am J Psych,* 146.5: 577-87.

Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC, and Husemann J. 2003. Adult mouse astrocytes degrade amyloid-β in vitro and in situ. *Nat Med,* 9(4):453-7

Xiao R. 2016. Levetiracetam might act as an efficacious drug to attenuate cognitive deficits of Alzheimer's disease. *Curr Top Med Chem*, 16:565-73.

Zare-shahabadi A, Masliah E, Johnson GVW, and Rezaei N. 2015. Autophagy in Alzheimer's Disease. *Rev Neurosci,* 26(4): 385–95.

Zhang MY, Zheng CY, Zou MM, Zhu JW, Zhang Y, Wang J. 2014. Lamotrigine attenuates deficits in synaptic plasticity and accumulation of amyloid plaques in APP/PS1 transgenic mice. *Neurobiol Aging*, 35:2713-25.

Zhao J, O'Connor T, Vassar R. 2011. The contribution of activated astrocytes to Aβ production: implications for Alzheimer's disease pathogenesis. *J Neuroinf,* 8:150.

Zheng WH, Bastianetto S, Mennicken F, Ma W, and Kar S. 2002. Amyloid β peptide induces tau phosphorylation and neuronal degeneration in rat primary septal cultured neurons. *Neuroscience,* 115:201-11.

Zheng XY, Zhang HY, Luo Q, and Zhu J. 2011. Kainic Acid-Induced Neurodegenerative Model: Potentials and Limitations. *J Biomed Biotech,* 2011:457079.