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Kainate Receptor Activation Enhances Amyloidogenic Processing of APP in Astrocytes

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Abstract

Kainic acid (KA) is an 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. However, biomolecular mechanisms responsible for the neuronal loss that occurs as a consequence of this treatment remains elusive. We have recently reported that toxicity induced by KA can partly be mediated by astrocyte-derived amyloid β (Aβ) peptides, which are critical in the development of Alzheimer's disease (AD). Nonetheless, little is known how KA can influence amyloid precursor protein (APP) levels and processing in astrocytes. Thus, in the present study using human U-373 astrocytoma and rat primary astrocytes, we evaluated the role of KA on APP metabolism. Our results revealed that KA treatment increased the levels of APP and its cleaved products (α-/β-CTFs) in cultured U-373 astrocytoma and primary astrocytes, without altering the cell viability. The cellular and secretory levels of $A\beta_{1-40}$/$A\beta_{1-42}$ were markedly increased in KA-treated astrocytes. We also demonstrated that the steady-state levels of APP-secretases were not altered but the activity of γ-secretase is enhanced in KA-treated U-373 astrocytoma. Furthermore, using selective receptor antagonists, we showed that the effects of KA is mediated by activation of kainate receptors and not NMDA or AMPA receptors. These results suggest that KA can enhance amyloidogenic processing of APP by activating its own receptor leading to increased production/secretion of Aβ-related peptides from activated astrocytes which may contribute to the pathogenesis of temporal lobe epilepsy.

Keywords Amyloid precursor protein · β-Amyloid · Epilepsy · Glial cells · Kainic acid

Introduction

Kainic acid (KA), a non-degradable analogue of the excitatory neurotransmitter glutamate, can induce seizures originating from the CA3 region of the hippocampus and then spread to other limbic structures. This is followed by loss of hippocampal neurons, reorganization of mossy fibers, and proliferation of both astrocytes and microglia, closely resembling the neuropathology characteristic of human mesial temporal lobe epilepsy (mTLE) [1–3]. The epileptogenic effects of KA are largely caused by the activation of hippocampal kainate receptors, resulting in aberrant synchronized glutamatergic currents that propagate seizures and facilitate neuronal loss [4, 5]. Some earlier studies have indicated that glutamate, originating from both neurons and astrocytes, may have an important role in triggering neurodegeneration following KA administration [6]. More recently, we have reported that KA-induced toxicity can partly be mediated by astrocyte-derived amyloid β (Aβ) peptides, which are critical in the development of Alzheimer's disease (AD) [7–9]. This is further supported by the evidence that Aβ peptides enhance the susceptibility of neurons to KA-induced toxicity [10] and that inhibiting Aβ synthesis can attenuate KA-triggered neurodegeneration [9]. Nevertheless, very little is known how KA can regulate the Aβ production/secretion that underlies the loss of neurons.

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Aβ peptides are a group of 39–43 amino acid-long hydrophobic peptides produced constitutively in the normal brain. These peptides are derived from the amyloid precursor protein (APP), which is known to be proteolytically processed by either non-amyloidogenic α-secretase or amyloidogenic β-secretase pathways. The α-secretase, facilitated by the enzymes such as disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) or ADAM17, cleaves APP within the Aβ domain, yielding soluble N-terminal APPα and a 10kD C-terminal fragment (α-CTF) that can be further processed by γ-secretase to generate $Aβ_{17–40}$/$Aβ_{17–42}$ fragments. On the other hand, the β-secretase pathway, mediated by the aspartyl protease β-site APP cleaving enzyme (BACE1), cleaves APP to generate soluble APPβ and an Aβ-containing C-terminal fragment (β-CTF), which is subsequently processed via γ-secretase to yield full-length $Aβ_{1–40}$/$Aβ_{1–42}$ peptides [11]. The γ-secretase itself comprises the aspartyl protease presenilin 1 or 2 (PS1/PS2) and three cofactors: nicastrin, presenilin enhancer 2 (PEN2), and anterior pharynx defective 1 (APH1) [11–13]. Although neurons are believed to be the major source of Aβ peptides, astrocytes that are activated in response to insults and pathological conditions such as mTLE also display higher levels of APP and/or its processing enzymes, which may contribute to the generation of Aβ [14–19]. APP processing is known to be influenced by a variety of factors, including the activation of certain ionotropic as well as metabotropic glutamatergic receptors (mGluR). Short treatments with *N*-methyl-D-aspartate (NMDA) receptor and mGluR1 agonists have been shown to increase intracellular accumulation of α-CTF and reduce generation of Aβ peptides. This is apparent both in cultured neurons as well as astrocytes which express mostly the Kunitz protease inhibitor-domain containing APP [20, 21]. Conversely, prolonged activation of NMDA receptors increases amyloidogenic APP processing [22, 23]. More recent data further suggest that while the activation of mGluR2 and mGluR5 is also involved with increased amyloidogenic processing of APP, direct stimulation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor increases α-secretase-mediated APP processing and inhibits Aβ production [24, 25]. In contrast to the aforementioned glutamate receptors, the role of kainate receptors in Aβ production has hitherto been unexplored. Considering the evidence that KA-induced neurodegeneration can partly be mediated by astrocyte-derived Aβ peptides [9], it is important to establish the significance of kainate receptor on APP processing in astrocytes. In the present study, using human glioblastoma U-373 and rat primary astrocytes, we showed that the activation of the kainate receptor by KA can increase the levels and processing of APP, leading to enhanced production/secretion of Aβ peptides. These results not only highlight the contribution of the kainate receptor in the production of Aβ peptides from astrocytes but also provide a basis to define a molecular pathway that may underlie the degeneration of neurons in mTLE brains.

Methods

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 Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum, 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 $Aβ_{1–40}$ and $Aβ_{1–42}$. High-sensitivity ELISA kits for the detection of human $Aβ_{1–40}$ and $Aβ_{1–42}$, on the other hand, were purchased from Millipore (Etobicoke, ON, Canada), along with Amicon Ultra-4 centrifugal filter columns. The enhanced chemiluminescence kit and bicinchoninic acid (BCA) protein assay kits were purchased from Thermo Fisher Scientific (Montreal, QC, Canada), whereas ADAM10 α-secretase activity assay kit was from AnaSpec (Fremont, CA, USA). Cycloheximide, KA, γ-secretase inhibitor DAPT, and 3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Oakville, ON, Canada), KA-receptor antagonist (*S*)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione (ACET) and AMPA-receptor 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 NMDA-receptor antagonist 2-amino-5-phosphonopentanoic acid (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 1. The associated horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz (Pasa Robles, CA, USA) and fluorescently coupled secondary antibodies were from Thermo Fisher. All other reagents were from either Sigma-Aldrich or Fisher Scientific.

U-373 Human Astrocytoma Culture and Treatments

U-373 cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum and maintained at 37 °C and 5% CO_2/air-humidified incubator. Cells were seeded at 1 ×

Table 1 List of primary antibodies

Antibody	Type	IF dilution	WB dilution	Source
ADAM10	Polyclonal	n/a	1:2000	EMD Millipore Co.
AMPA receptor	Polyclonal	1:1000	n/a	Abcam Inc.
APH1	Polyclonal	n/a	1:1000	Abcam Inc.
APP (clone Y188)	Monoclonal	1:100	1:5000	Abcam Inc.
APP-KPI	Polyclonal	n/a	1:1000	Abcam Inc.
APP (22C11)	Monoclonal	1:2000	n/a	EMD Millipore Co.
BACE1	Polyclonal	n/a	1:2000	Abcam Inc.
β-Actin	Monoclonal	n/a	1:5000	Sigma-Aldrich, Inc.
β-Amyloid, 17–24 (4G8)	Monoclonal	1:250	n/a	Covance Corp.
GFAP	Polyclonal	1:1000	n/a	Abcam Inc.
KA receptor	Polyclonal	1:2000	n/a	Sigma-Aldrich, Inc.
Nicastrin	Polyclonal	n/a	1:800	Santa Cruz Biotechnology
NMDA receptor	Monoclonal	1:100	n/a	Thermo Fisher Scientific
PEN2	Polyclonal	n/a	1:2000	EMD Millipore Co.
PS1	Polyclonal	n/a	1:2000	EMD Millipore Co.

IF immunofluorescence, *WB* Western blotting, *n/a* not used in specified application

10^4 cells/cm^2 and the medium was replaced every 2–3 days. For experiments, cells were plated on six-well plates at 1×10^4 cells/cm^2 for 24 h and grown to confluency. Media was replaced prior to treatment with any reagents. In a series of experiments, U-373 cells were first treated with different concentrations (10 μM, 100 μM, 1 mM, or 10 mM) of KA over a range of time points (3, 6, 12, 24, and 48 h). Using this protocol, we ascertained that 100 μM KA for 24-h treatment is the most optimal condition and hence this concentration of KA was used in all subsequent experiments. In some studies, U-373 cells were first treated with either 100 μM KA or saline for 24 h, and then exposed to 30 μg/ml cycloheximide for 0.5, 1, 2, or 4 h [26]. In parallel, U-373 cells were treated with 100 μM KA for 24 h in the presence or absence of 100 μM ACET, 100 μM AP5, or 20 μM GYKI 52466. The concentrations used for ACET, AP5, and GYKI 52466 were based on previous results [27–29]. After various experiments, cells were harvested and then processed for Western blotting, immunocytochemistry, enzyme activity, ELISA, or cell viability assays.

Rat Astrocyte Cultures and Treatment

Primary rat hippocampal astrocytes (Sciencell, CA, USA) were seeded on poly-D-lysine-coated plates and grown in the associated 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 six-well plates at 1×10^4 cells/cm^2 for 24 h and grown to confluency. Media was replaced prior to treatment with any drug/substance. In a series of experiments, cultured astrocytes were treated with 100 μM KA for different periods of time (3, 6, 12, 24, and 48 h) and then processed for Western blotting, ELISA, or cell viability assays.

Western Blotting

U-373 and primary astrocyte cultured cells from various experiments 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 or NuPAGE 4–12% Bis–Tris gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes, blocked with 5% milk, and incubated overnight at 4 °C with anti-APP recognizing N-terminal fragment (22C11), anti-APP recognizing C-terminal fragment (Y188), anti-APP recognizing Kunitz family of serine protease inhibitor (APP-KPI), anti-ADAM10, anti-BACE1, anti-PS1, anti-nicastrin, anti-PEN2, or anti-APH1 antisera at dilutions listed in Table 1. The following day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) and immunoreactive proteins were detected with enhanced chemiluminescence kit. All blots were re-probed with anti-β-actin antibody and quantified using a MCID image analysis system [30].

Immunocytochemistry

To establish the presence of glutamatergic NMDA, AMPA, and KA receptors, control U-373 cells were processed for immunocytochemistry as described earlier [30]. In parallel, we evaluated cellular localization of APP and its cleaved products in control and KA-treated cultured U-373 cells. In brief, U-373 cells were seeded at 1×10^4 cells/cm^2 on coverslips in a 24-

well plate, fixed with 4% paraformaldehyde (PFA), and then incubated overnight at 4 °C with either anti-NMDA, anti-AMPA, or anti-KA receptor antisera in combination with anti-glial fibrillary acidic protein (GFAP) antisera at dilutions listed in Table 1. In another series of experiments, U-373 cells were treated with or without 100 μM KA for 24 h, fixed with 4% PFA and then incubated overnight at 4 °C with anti-APP (N- and C-terminal) or anti-Aβ antibodies in combination with anti-GFAP antisera at dilutions listed in Table 1. The coverslips were then exposed to Alexa Fluor 488/594-conjugated secondary antibodies (1:250), stained with DAPI, and mounted with ProLong Gold antifade medium. Immunostained cells were visualized using a Zeiss multiphoton confocal laser scanning microscope (LSM 510; Carl Zeiss, Inc.) equipped with a ×40 Plan-apochromatic oil-immersion lens (1.2 NA).

Secretase Activity Assays

Control and KA-treated cultured U-373 cells were processed to measure α-secretase ADAM10 or β-secretase BACE1 activity according to the manufacturer's instructions using the respective assay kit, as described earlier [31]. The fluorescence was measured at excitation wavelength of 355 nm and emission wavelength of 495 nm. The γ-secretase activity was measured on crude membrane fractions. Briefly, control and treated cells were lysed using sample buffer (10 mM Tris base, 1 mM EDTA, 1× 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 protein content was determined using BCA. The γ-secretase activity was then measured in 50 mM Tris–HCl (pH 6.8), 2 mM EDTA, and 0.25% CHAPSO with 8 μM fluorogenic γ-secretase substrate in 50 μg protein [32]. The fluorescence was detected at excitation wavelength of 355 nm and emission wavelength of 440 nm and the specificity was determined with 100 μM γ-secretase inhibitor L-658458. All samples were assayed in duplicate and results were obtained from four independent experiments.

ELISA for $A\beta_{1-40/1-42}$

To measure cellular Aβ levels, control and KA-treated cultured U-373 cells were solubilized in RIPA buffer, centrifuged, and then assayed for human $A\beta_{1-40}$ and $A\beta_{1-42}$ using respective human ELISA kits as described earlier [30, 31]. For the measurement of secreted $A\beta_{1-40/1-42}$, conditioned media collected from control and KA-treated cells were concentrated using Amicon filtration columns with 3-kDa MW cut-off and then analyzed using the ELISA kits. The absorbance was read with a microplate reader, and the amount of $A\beta_{1-40/1-42}$ in each sample was calculated from the standard curve. The levels of cellular and secreted $A\beta_{1-40/1-42}$ peptides in rat primary astrocyte cells treated with or without 100 μM KA were also similarly measured with commercial rat/mouse ELISA kits [31]. All samples were assayed in duplicate and results presented were obtained from four independent experiments.

Cell Viability Assays

The viability of cultured U-373 cells exposed to different concentrations (100 μM to 10 mM) of KA for 24 h or primary astrocytes exposed to 100 μM KA for 24 h was assessed using the MTT assay as described earlier [32]. In brief, control and treated culture plates were replaced with new medium containing 0.25% MTT and then incubated for 2 h at 37 °C. The reaction was terminated and measured spectrophotometrically at 570 nm. The experiments were performed in quadruplicate and results presented were obtained from four independent experiments.

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, whereas for 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 was performed using GraphPad Prism (GraphPad Software, Inc., CA, USA).

Results

Effects of KA 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 [30, 33]. To characterize the potential effects of KA on APP metabolism in astrocytes, we first evaluated APP holoprotein levels in cultured U-373 cells following exposure to different concentrations (10 μM, 100 μM, and 1 mM) of KA for varying lengths (0–48 h) of time. Our results clearly revealed a significant time-dependent increase in APP levels as a consequence of treatment with different doses of KA (Fig. 1a–c). A significant increase in APP level was evident at 3 h with 10 μM KA, and 6-24 h with 100 μM KA as well as 1 mM KA. The upregulation of APP was most prominent at 24 h following treatment with 1 mM KA, reaching ~175% of the control levels before declining by 48 h of treatment (Fig. 1a–c). The observed increase in APP levels is evident not only with antibodies that label APP C- and N-terminal fragments (22C11 and Y188) (Fig. 1a–c) but also the KPI-domain containing

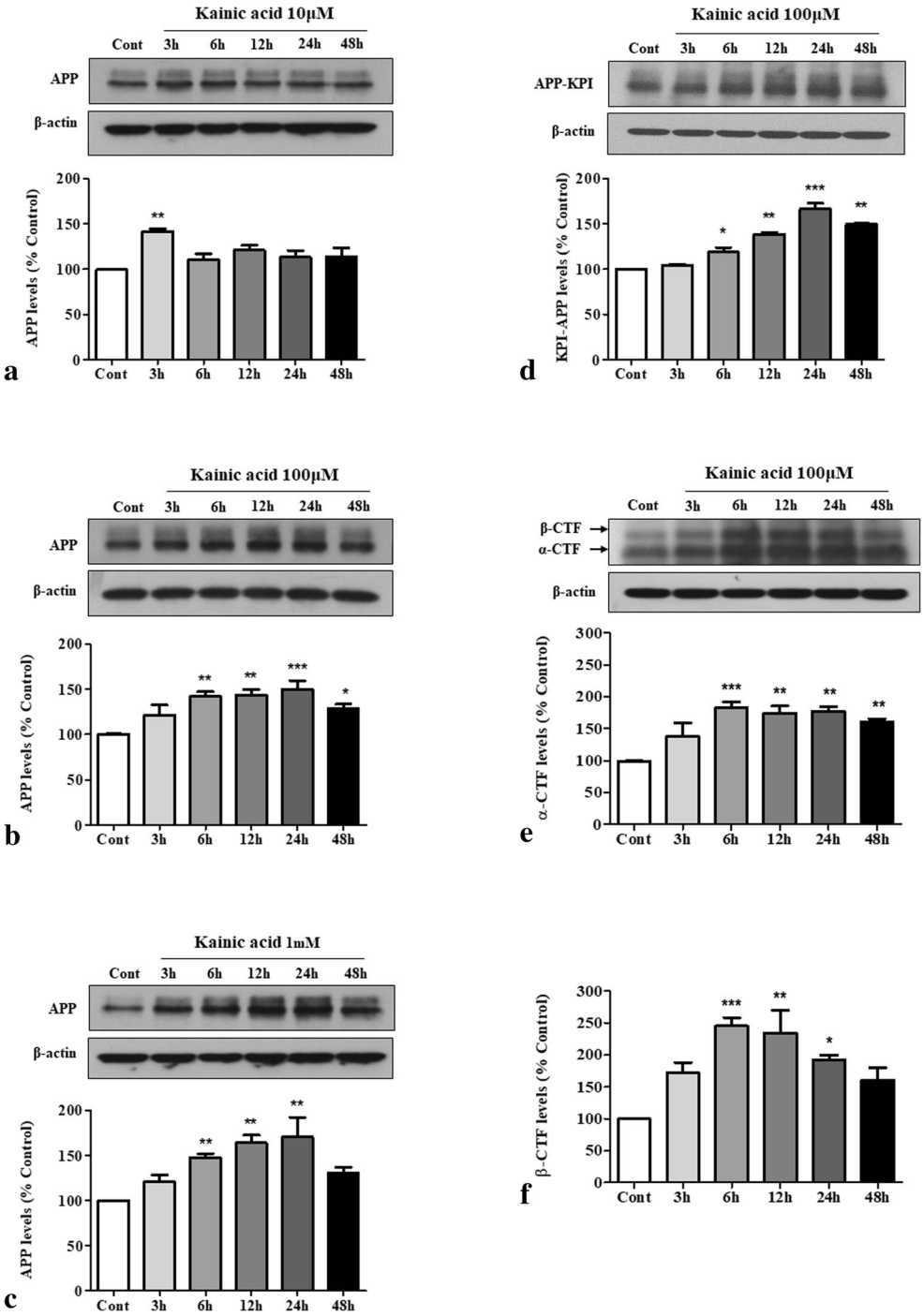

Fig. 1 **a–f** Immunoblots and corresponding histograms showing dose- and time-dependent effects of kainic acid (KA) on the levels of APP holoprotein (detected by Y188 monoclonal antibody against C-terminal APP fragment) and APP-CTFs (i.e., α-CTF and β-CTF) in cultured U-373 cells. Note the selective increase in the levels of APP (**a–d**) as well as α-CTF (**e**) and β-CTF (**f**) following treatment with KA. All Western blots were re-probed with β-actin antibody to monitor protein loading. Data represent means ± SEM from 3 to 4 independent experiments. Cont, control; KA, kainic acid. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

APP known to be expressed mostly in astrocytes (Fig. 1d). In parallel to APP, we analyzed the levels of α- and β-secretase cleavage products α-CTF and β-CTF, respectively, following exposure to KA for different periods of time. Our results clearly indicate that 100 μM KA induced an increase in both α-CTF/β-CTF levels between 6 and 24 h post-treatment and then it declined by 48 h as observed with APP levels (Fig. 1e, f). Although the levels of both CTFs increased following KA treatment, the magnitude of change was found to be much more prominent for β-CTF (i.e., ~ 250% of the control) compared to α-CTF (i.e., ~ 175% of the control).

To assess whether altered levels of α-/β-CTFs rely on altered expression of the respective secretases or on the regulation of their enzymatic activities, we first evaluated the steady-state levels of α-secretase ADAM10 (Fig. 2a), β-secretase BACE1 (Fig. 2b), and components of the γ-secretase complex including PS1 (Fig. 2c), nicastrin, PEN2, and APH1 (Supplementary Fig. 1) in U-373 cells following exposure to

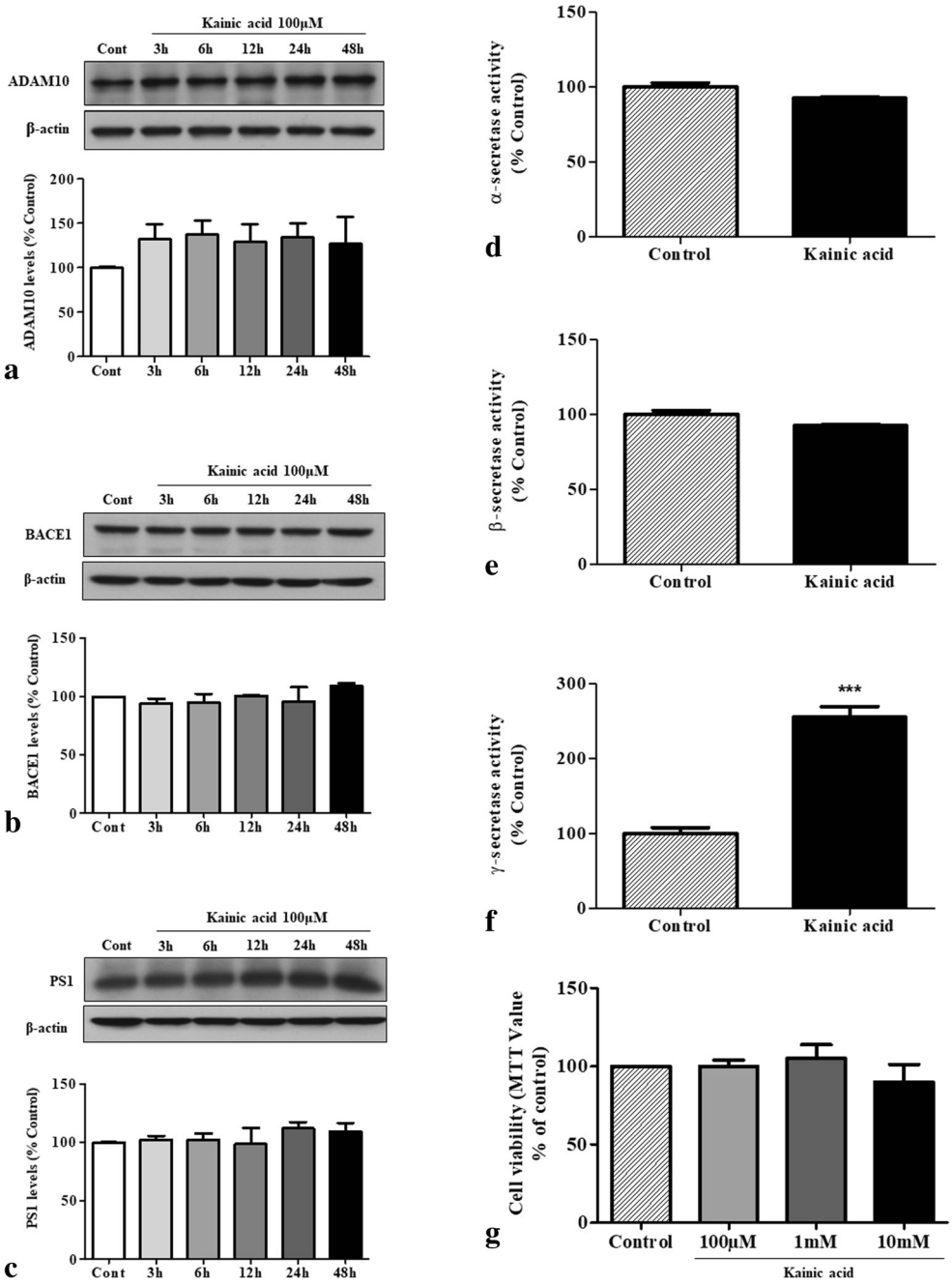

Fig. 2 **a–c** Immunoblots and corresponding histograms showing time-dependent effects of 100 μM kainic acid (KA) on the levels of α-secretase ADAM10 (**a**), β-secretase BACE1 (**b**), and components of γ-secretase complex PS1 (**c**) in cultured U-373 cells. Note that KA treatment did not alter the steady-state levels of either ADAM10, BACE1, or PS1 in U-373 cells. **d–f** Histograms depicting unaltered activity of α-secretase ADAM10 (**d**) and β-secretase BACE1 (**e**), but increased activity of γ-secretase complex (**f**) following treatment with 100 μM KA for 24 h. All Western blots were re-probed with β-actin antibody to monitor protein loading. Data represent means ± SEM from 3 to 4 independent experiments. Cont, control; ***$p < 0.001$

100 μM KA over a period of 3–48 h. Interestingly, our results revealed no significant changes in either ADAM10, BACE1, or any of the four components of the γ-secretase complex, i.e., PS1, nicastrin, APH1, or PEN2, at any time point (Fig. 2a–c; Supplementary Fig. 1). Considering the evidence that steady-state levels of BACE1 or γ-secretase complex often does not correspond with enzyme activity [11, 13], we subsequently assessed changes in the activity of these three secretases. While no marked change was apparent in the activity of either ADAM10 (Fig. 2d) or BACE1 (Fig. 2e) in cells treated 100 μM KA for 24 h, the activity of γ-secretase increased threefold in KA-treated cells compared to control cells (Fig. 2f), thus suggesting that KA may lead to an increased production of Aβ peptides, at least in part, by modulating the activity of this crucial enzyme. Additionally, this effect of KA is evident without any alteration in the viability of cultured U-373 cells (Fig. 2g). To determine whether the enhanced levels of APP and APP-CTFs are the consequence of decreased turnover, cultured U-373 cells were treated with or without 100 μM KA for 24 h and then exposed to cycloheximide for different periods of time (i.e., 0, 0.5, 1, 2, and 4 h) to inhibit de novo synthesis of proteins [34, 35]. As expected, our results clearly showed that levels of APP and α-CTF/β-CTF were markedly higher in U-373 cells treated with cycloheximide,

but these peptides decline as a function of time without any significant difference between KA-treated and untreated control cells (Fig. 3a–c).

Effects of KA on Aβ Levels/Secretion in U-373 Cells

To determine whether the KA-induced alteration in 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 cell lysates and conditioned media of U-373 cells treated with or without 100 μM KA for 24 h. 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 an increased production as well as secretion of these peptides (Fig. 4a–d).

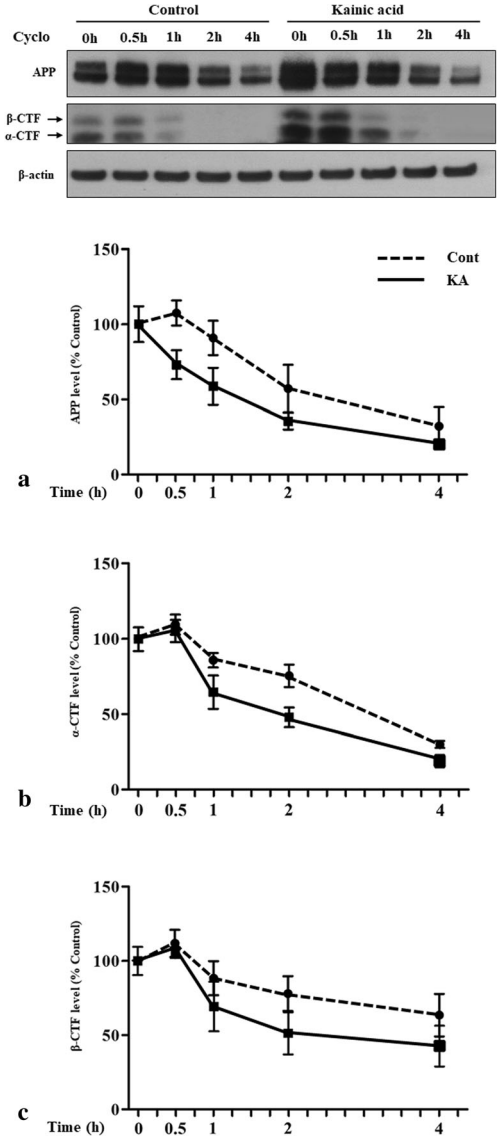

Fig. 3 **a–c** Immunoblots and corresponding histograms showing the levels of APP, α-CTF, and β-CTF in cultured U-373 cells with or without 100 μM kainic acid (KA) treatment for 19 h followed by exposure to 30 μg/ml cycloheximide for a period of up to 4 h. Note the relative levels of APP holoprotein (**a**), α-CTF (**b**), and β-CTF (**c**) in control and KA-treated cells in the presence of cycloheximide. All Western blots were re-probed with β-actin antibody to monitor protein loading. Data represent means ± SEM from 3 to 4 independent experiments

Effects of KA Antagonist ACET on APP Metabolism

To determine whether the effects of KA on APP metabolism are mediated by selective activation of KA receptors, we first evaluated the presence of KA, NMDA, and AMPA receptors in cultured U-373 cells using immunocytochemistry. Our results clearly reveal the presence of KA, AMPA, and NMDA receptors in cultured astrocytes as reported earlier [36–38] (Supplementary Fig. 2). Subsequently, U-373 cells were treated with 100 μM KA in the presence or absence of KA receptor antagonist ACET (100 μM) for 24 h and then processed to evaluate the levels of APP and its cleaved products. This antagonist has previously been shown to selectively block the response of KA [28]. It is apparent from our results that while ACET itself did not alter the levels of APP or α-CTF/β-CTF, it was able to significantly attenuate the effects of KA on APP holoprotein and its metabolites (Fig. 4e–g). Additionally, potentiation of cellular and secretory levels of $A\beta_{1–40}$ and $A\beta_{1–42}$ observed following exposure to KA was found to be attenuated by ACET treatment (see Fig. 4a–d). In contrast to ACET, neither NMDA receptor antagonist AP5 (Fig. 5a–c) nor AMPA receptor antagonist GYKI-54266 (Fig. 5d–f) was able to alter the effects of KA on the levels of APP or its metabolites.

Effects of KA on Cellular Localization of APP and its Metabolites

To validate our Western blot data, we assessed the cellular localization of APP, APP-CTFs, and Aβ in control and KA-treated U-373 cells using confocal microscopy (Fig. 6). Full-length APP was localized using N-terminal monoclonal antibody 22C11 and the localization of APP/APP-CTFs was determined with C-terminal monoclonal antibody Y188. Previously, we showed that Y188 immunoreactivity overlaps completely with that of a C-terminal APP antibody CTM1 but only partially with the immunoreactivity detected by 22C11 [32]. The monoclonal antibody 4G8 which specifically recognizes Aβ without reacting with APP or APP-CTFs [39, 40] was used to label Aβ. Immunoreactivities detected using 22C11 (Fig. 6a, b), Y188 (Fig. 6e, f), and 4G8 (Fig. 6i, j) antibodies exhibited perinuclear localization and punctate staining throughout the cytoplasm in untreated U-373 cells. Exposure to 100 μM KA for 24 h did not alter distributional profile but intensified immunoreactivity noted with 22C11 (Fig. 6a–d), Y188 (Fig. 6e–h), and 4G8 (Fig. 6i–l) antibodies, compared to control U-373 cells.

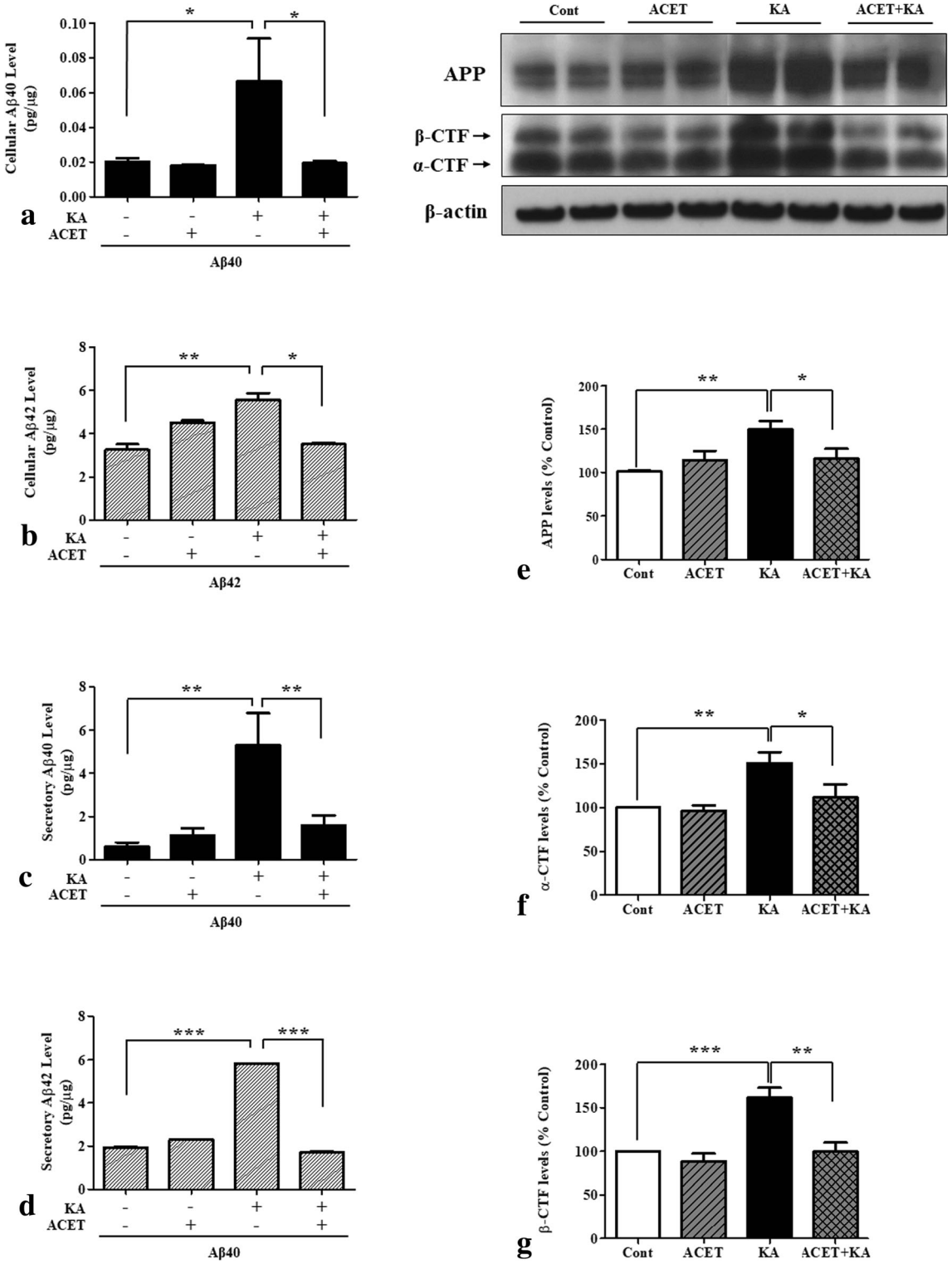

Fig. 4 **a–d** Histograms showing the effects of 100 μM kainic acid (KA), 100 μM ACET (KA antagonist), and 100 μM KA + 100 μM ACET treatment for 24 h on cellular (**a, b**) and secretory (**c, d**) levels of $A\beta_{1-40}$ (**a, c**) and $A\beta_{1-42}$ (**b, d**) in cultured U-373 cells. Note the significant increase in cellular and secretory levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$ in KA-treated U-373 cells and its attenuation following exposure to ACET. **e–g** Immunoblots and corresponding histograms showing the effects of 100 μM KA, 100 μM ACET, and 100 μM KA + 100 μM ACET treatment for 24 h on the levels of APP (**e**) and APP-CTFs (i.e., α-CTF and β-CTF) in cultured U-373 cells. Note the effects of ACET in attenuating KA-induced increased levels of APP (**e**), α-CTF (**f**), and β-CTF (**g**) in U-373 cells. All Western blots were re-probed with β-actin antibody to monitor protein loading. Data represent means ± SEM from 3 to 4 independent experiments. Cont, control; KA, kainic acid. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

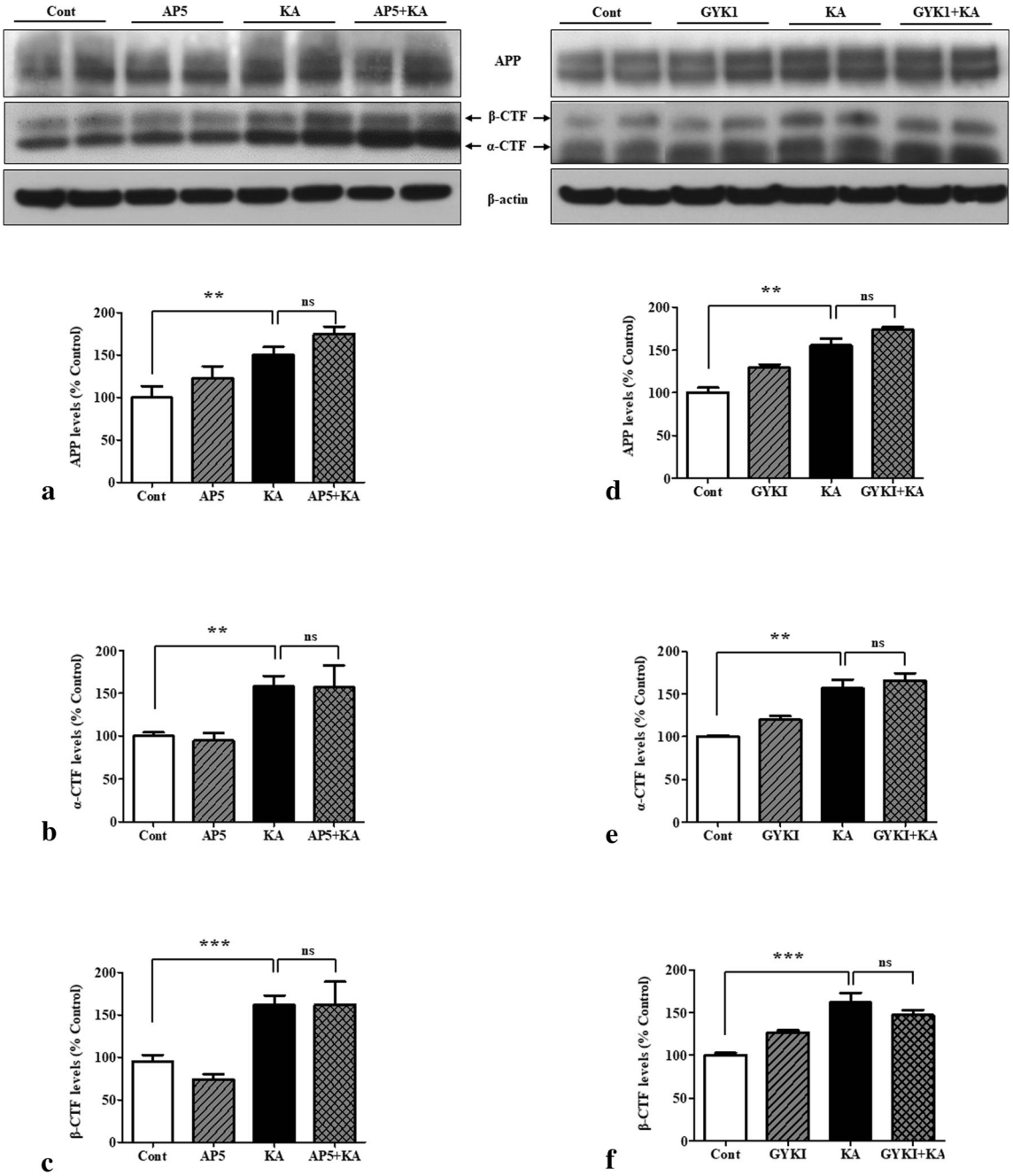

Fig. 5 **a**–**c** Immunoblots and corresponding histograms showing the effects of 100 μM kainic acid (KA) for 24 h in the absence and presence of glutamatergic NMDA receptor antagonist AP5 in cultured U-373 cells. Note that AP5 did not affect KA-induced increased levels of APP (**a**), α-CTF (**b**), and β-CTF (**c**) in U-373 cells. **d**–**f** Immunoblots and corresponding histograms showing the effects of 100 μM KA for 24 h in the absence and presence of glutamatergic AMPA receptor antagonist GYK1 in cultured U-373 cells. Note that GYK1 did not affect KA-induced increased levels of APP (**a**), α-CTF (**b**), and β-CTF (**c**) in U-373 cells. All Western blots were re-probed with β-actin antibody to monitor protein loading. Data represent means ± SEM from 3 to 4 independent experiments. Cont, control; KA, kainic acid; ns, non-significant. $^{**}p < 0.01$, $^{***}p < 0.001$

Effects of KA on APP Metabolism in Rat Primary Astrocytes

In order to confirm that the effects of KA observed in glioblastoma U-373 cells occur in primary astrocytes, rat hippocampal astrocytes were treated with 100 μM KA for different periods of time (3–48 h) and then assessed to measure the levels of APP holoprotein and its metabolites. In agreement with our U-373 cells, the levels of APP, α-CTF as well as β-CTF were markedly increased between

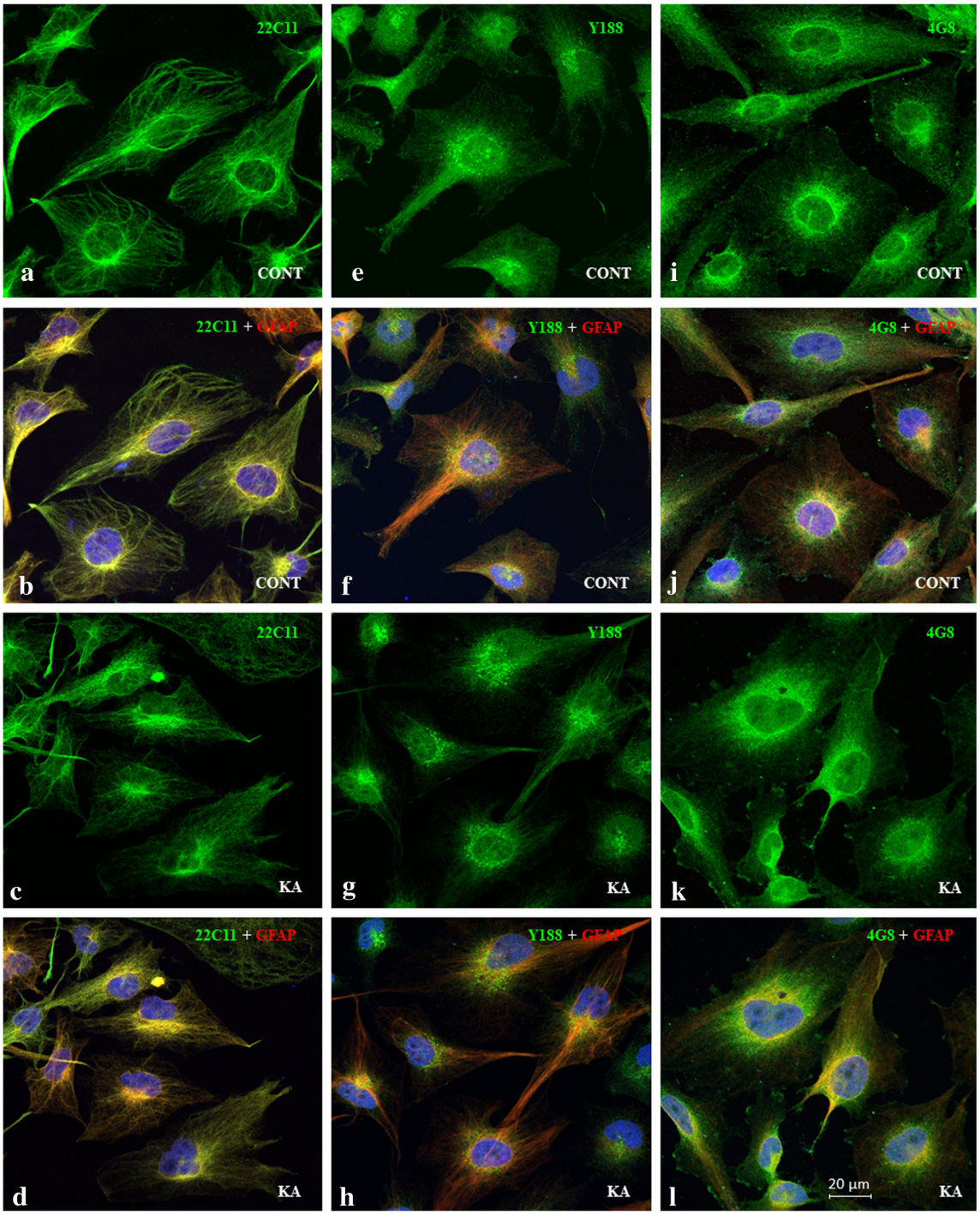

Fig. 6 **a–l** Representative confocal images showing cellular distribution of immunoreactive APP and GFAP (**a–d**), APP/APP-CTFs and GFAP (**e–h**), and Aβ and GFAP (**i–l**) in control (**a, b, e, f, i, j**) and kainic acid (KA)–treated (**c, d, g, h, k, l**) cultured U-373 cells. Note the increased expression of APP, CTFs, and Aβ peptide in KA-treated cells compared to control cells. Identity of primary antibodies is indicated by respective font colors. Cont, control; KA, kainic acid. Scale = 20 μm

12 and 24 h in KA-treated astrocytes compared to control astrocytes (Fig. 7a–c). Additionally, we did not observe any alteration in the steady-state levels of either BACE1 or PS1 levels at any time point following KA treatment (Supplementary Fig. 3).

Effects of KA on Aβ Levels/Secretion in Rat Primary Astrocytes

To determine if changes in the levels of APP and CTFs can lead to increased levels of Aβ peptides, rat primary

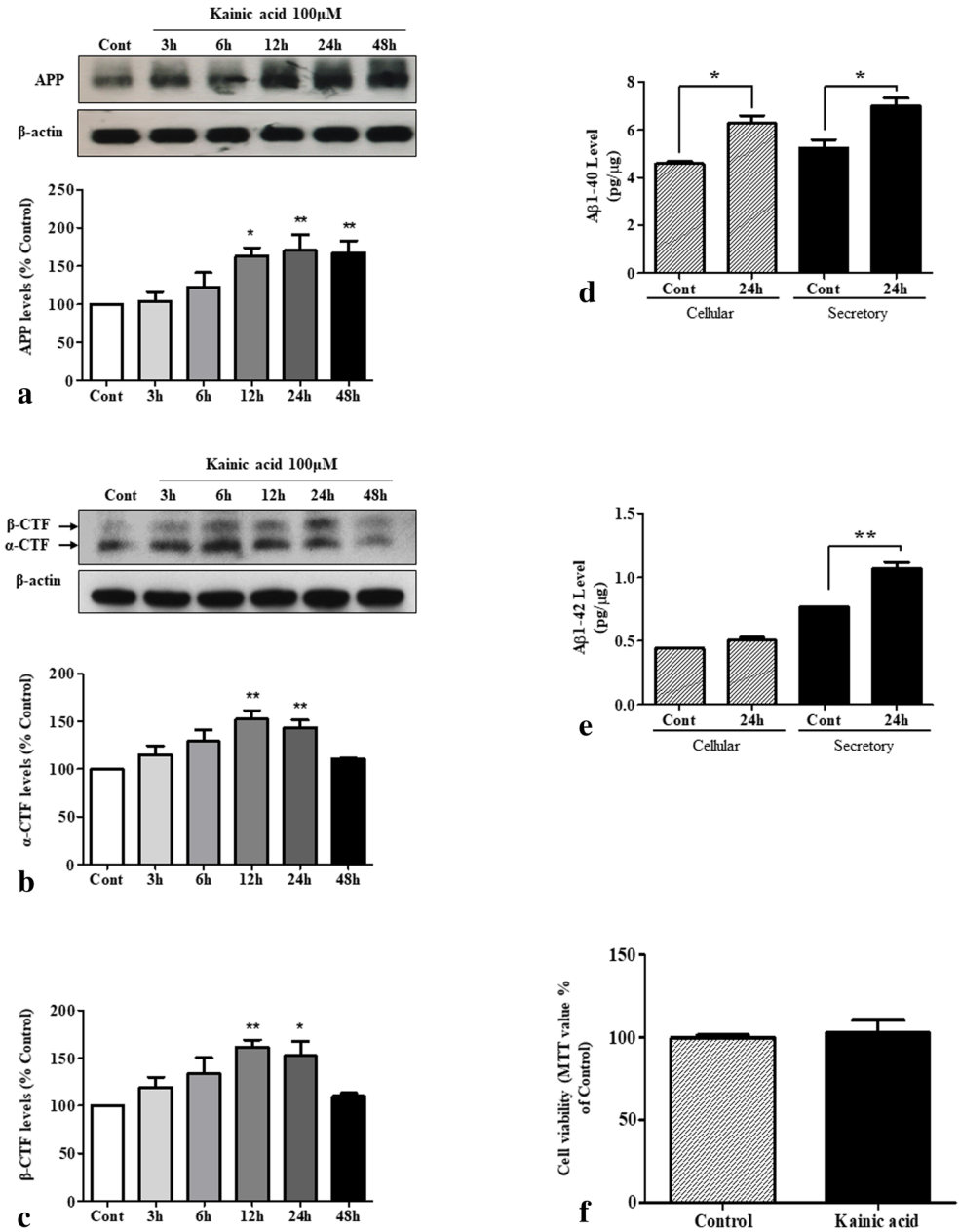

Fig. 7 **a–c** Immunoblots and corresponding histograms showing time-dependent effects of 100 μM kainic acid (KA) on the levels of APP holoprotein (**a**) and APP-CTFs (i.e., α-CTF and β-CTF) (**b**, **c**) in rat primary hippocampal cultured astrocytes. Note the selective increase in the levels of APP as well as α-CTF and β-CTF following treatment with KA. **d**, **e** Histograms showing the effects of 100 μM KA treatment for 24 h on cellular and secretory levels of $A\beta_{1–40}$ (d) and $A\beta_{1–42}$ (e) in cultured astrocytes. Note the significant increase in cellular and secretory levels of Aβ peptides in KA-treated primary astrocytes. **f** Histogram depicting that viability of cultured rat astrocytes, as evident by MTT assay, was not altered following treatment with 100 μM KA for 24 h. All Western blots were re-probed with β-actin antibody to monitor protein loading. Data represent means ± SEM from 3 to 4 independent experiments. Cont, control; KA, kainic acid. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

astrocytes were treated with 100 μM KA for 24 h and then processed to measure cellular/secretory $A\beta_{1–40}$/$A\beta_{1–42}$ levels using ELISA. Our results showed that cellular levels of $A\beta_{1–40}$ were significantly increased at 24 h, whereas levels of $A\beta_{1–42}$ did not reach significance at 24 h after KA treatment (Fig. 7d, e). The secretory levels of $A\beta_{1–40}$ and $A\beta_{1–42}$, however, were markedly increased at 24 h following exposure to 100 μM KA (Fig. 7d, e). In parallel, we noted that the viability of cultured astrocytes, as observed with U-373 cells, was not compromised after 24 h exposure to 100 μM KA (Fig. 7f).

Discussion

The results obtained in this study suggest that KA can increase the level and processing of APP by activating its own receptor, leading to the increased production/secretion of Aβ-related peptides in astrocytes. This is supported by data which show that (1) KA triggers a time- and dose-dependent increase of APP expression in human cultured U-373 glioblastoma cells; (2) this increase is accompanied by a corresponding upregulation of APP-cleaved products, including α-/β-CTF intermediates, as well as $A\beta_{1–40}$/$A\beta_{1–42}$ peptides; (3) the changes in APP/Aβ metabolism following KA treatment are mediated by

the kainate receptor, as opposed to the glutamatergic NMDA or AMPA receptors; (4) KA treatment of rat primary hippocampal astrocytes also results in increased levels of APP, APP-CTFs, and enhanced production/secretion of $A\beta_{1-40}/A\beta_{1-42}$ peptides. Since KA treatment can lead to neuropathological changes resembling those observed in human mTLE, it is possible that APP/Aβ peptides derived from astrocytes may have a role in mTLE pathogenesis.

Astrocytes are the most abundant glial cells in the central nervous system. They play vital roles in maintaining brain homeostasis by regulating trophic/metabolic support, neurotransmitter milieus, blood–brain barrier integrity, synaptic activity, and synapse formation/remodeling [41–46]. Normal astrocytes, unlike neurons, express very little APP and its processing enzymes, particularly BACE1 and the γ-secretase components [47–50]. Thus, neuronal cells appear to be the primary source for Aβ in physiological conditions, whereas astrocytes fulfill a partial role in the clearance and degradation of extracellular Aβ peptides [19, 51]. Upon activation, which may result from injury or development of 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 [42, 43, 45, 46, 52–57]. Concurrently, activated astrocytes have been shown to express APP, BACE1, and PS1 under certain experimental conditions, such as cerebral ischemia, traumatic brain injury, excitotoxicity, and cholesterol sequestration [15, 17, 58–62]. 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 [14, 18, 63, 64]. Hence, it is possible that the activation of astrocytes under certain experimental/pathological conditions may cause these cells to produce Aβ-related peptides which can contribute to the loss of neurons and/or in the development of disease pathology.

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 pyramidal neurons [8, 61, 65]. It is unclear, however, whether this effect was mediated by direct activation of astrocytic kainite receptors or indirectly via other mechanisms. The present study using U-373 cells reveals that KA treatment can enhance the levels of APP holoprotein, including the isoform containing KPI-domain known to be expressed mostly in astrocytes, in a time-dependent manner. This is accompanied by an increased level of α-/β-CTFs as well as intracellular/secretory levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides. Interestingly, we did not observe any alteration in the steady-state levels of the APP secretases, but the activity of γ-secretase is increased in KA-treated cultured U-373 cells. The discrepancy between the steady-state levels of γ-secretase complex and activity of this enzyme has been reported earlier [30] 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 [66, 67]. The observed changes in the APP holoprotein and its metabolites are evident in absence of any alterations in cell viability, suggesting that it is not due to the consequence of kainite-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 $A\beta_{1-40}/A\beta_{1-42}$ are likely the consequence of increased production as opposed to decreased clearance of the peptides.

Astrocytes, as observed in the present study, are known to express glutamatergic NMDA, AMPA, and kainate receptors [36–38], but their pharmacological profile in relation to interaction with kainic acid remains unclear. Some earlier studies using either brain tissues and cultured neurons reported that kainic acid can compete for NMDA and AMPA receptors at ~ 10–100 times lower affinity than the kainate receptors [68–71]. There is also evidence that 30 μM kainic acid can inhibit ~ 20% of L-[^{3}H-glutamate] binding in human temporal cortex [72]. At present, to what extent 100 μM kainic acid can interact with NMDA or AMPA receptors vis a vis kainate receptors in cultured astrocytes remains unclear. Nevertheless, our data showing that the effects of KA on APP metabolism in cultured astrocytes is inhibited by the kainate receptor antagonist ACET, but not by either a selective NMDA receptor antagonist AP5 or a selective AMPA receptor antagonist GYKI-52466, suggest that the selective activation of kainate receptors may possibly underlie increased levels and processing of APP in cultured astrocytes. It is also of interest to note that most of the results observed in human glioblastoma were also replicated in rat primary astrocytes, indicating that a similar phenomenon may occur in both humans and rat models. Given the fact that astrocytes vastly outnumber neurons in the brain [51], our results show that activated astrocytes may serve as a potential source of Aβ under certain pathological conditions. Nevertheless, future experiments are needed to define the underlying mechanism by which KA treatment can lead to increased levels of APP holoprotein and its cleaved products including Aβ peptides in astrocytes.

Administration of KA has been shown to reproduce some of the hallmark features of mTLE, including the recurrent seizures, synaptic reorganization, widespread gliosis, and neuronal loss in the CA1/CA3 regions of the hippocampus [3, 73–75]. Excitotoxic glutamatergic transmission resulting from neuronal hyperactivity has been suggested to play a role in triggering seizures and death of neurons in KA models of epilepsy. By regulating the uptake and release of glutamate,

activated astrocytes may also simultaneously contribute to the generation of seizures and loss of neurons in the hippocampus [76–78]. Since Aβ peptides can potentiate the release [79–81] and inhibit the uptake of glutamate by astrocytes [82, 83], it is likely that KA-induced Aβ production/secretion from activated astrocytes may play a role in the loss of neurons and/or development of disease pathology. It is, however, of interest to note that the kainic acid–induced increase in γ-secretase activity by regulating substrates other than APP [84, 85], not investigated in the current study, can also influence cell death and/or disease pathology. Nevertheless, a role for Aβ peptides derived from APP is supported by the evidence that (1) Aβ peptide can induce neuronal hyperexcitability and trigger epilepsy [86], (2) transgenic mice overexpressing Aβ peptide exhibit spontaneous as well as induced seizures more frequently than wild-type mice [87–89], (3) the prevalence of seizures is higher in AD cases than in control population [90–93], (4) antiepileptic drugs such as levetiracetam has been shown to partially reverse AD-related pathology [94–97], and (5) KA-induced neurotoxicity can be attenuated by inhibiting Aβ synthesis [8]. Additionally, it is reported that immunizing mutant APP transgenic mice against Aβ protects them from seizures [98]. These results, together with the present study, raise the possibility that KA can enhance the level/processing of APP by activating its own receptor leading to increased production of Aβ-related peptides from activated astrocytes which can subsequently influence the development of mTLE pathology.

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Author Contributions D.O. and A.S. performed experiments, analyzed data, and wrote the manuscript. S.K. conceived the study, analyzed data, and wrote the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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