The Potential Role of Naproxen in Promoting Rivastigmine Effect Against Aluminum Chloride-Induced Alzheimer-Like Model in Rats

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Abstract

Alzheimer's disease (AD) is one of the leading causes of dependence and disability among the elderly worldwide. The traditional anti-Alzheimer medication, rivastigmine, one of the cholinesterase inhibitors (ChEIs), fails to achieve a definitive cure. Neuroinflammation plays a central role in AD pathogenesis. We tested the hypothesis that naproxen, a non-steroidal anti-inflammatory drug (NSAID), administration to the rivastigmine-treated aluminum chloride (AlCl3), Alzheimer's rat model, could provide an additive neuroprotective effect compared to rivastigmine alone. The studied groups were control (Cont), $AlCl_3$ treated (Al), rivastigmine treated (RIVA), naproxen treated (Napro), and combined rivastigmine and naproxen treated (RIVA + Napro). Rats' memory, spatial learning, and cognitive behavior were assessed followed by evaluation of hippocampal acetylcholinesterase (AChE) activity. Hippocampal and cerebellar histopathology were thoroughly examined. A marker of astroglial injury; glial fibrillary acidic protein (GFAP), the apoptosis maker; activated caspase-3 and the neuroepithelial stem cells marker; nestin expressions were immunohistochemically assayed. AD rats displayed significantly impaired memory and cognitive function, augmented hippocampal AChE activity, massive neurodegeneration associated with enhanced astrogliosis, apoptosis, and impaired neurogenesis. Rivastigmine, naproxen, and their combination decreased hippocampal AChE activity, mitigated behavioral and neuropathological changes in Al-intoxicated rats, possibly through downregulation of activated caspase-3 and upregulation of nestin. Except for the enhancement of neurogenesis and suppression of apoptosis, the combination therapy had no additional neuroprotective benefit over rivastigmine-only therapy. Naproxen's efficacy was demonstrated by its ability to act at the cellular level, enhance neurogenesis, and suppress apoptosis without having an additional mitigating impact in Al-induced cognitive impairment.

1. Introduction

Alzheimer's disease (AD), a devastating age-related neurodegenerative disorder, is considered the most common cause of progressive dementia in the elderly above 65 (Reddy et al. 2020). It is manifested by memory impairment besides behavioral and personality changes and various cognitive deficits (Reddy et al. 2020). AD imposes a severe financial, mental and physical burden upon patients and their caregivers (Leifer 2003). Early intervention can help the preservation of neural structures; delay the onset of dementia and disease progression.

The neuropathological changes of the AD brain include intracellular neurofibrillary tangles (NFTs) of hyper-phosphorylated tau, extracellular amyloid plaques, and vascular deposits (Villemagne et al. 2013). They were particularly identified in the hippocampus, cerebral cortex (Villemagne et al. 2013), the molecular, granular, and Purkinje cell layers of the cerebellar cortex (Singh-Bains et al. 2019). Amyloid-β (Aβ) proteins, the principal component of senile plaques, play a crucial role in neurodegeneration (Nhan et al. 2015). Accumulating evidence reveals the critical role of Aβ-induced caspase-3 activation in neuronal apoptosis (Kim et al. 1997; Harada and Sugimoto 1999; Allen et al. 2001) still before amyloid plaques and NFTs formation (LaFerla et al. 1997; Guo et al. 1998).

Neurofibrillary degeneration of forebrain cholinergic neurons, a significant characteristic of AD, contributes to memory and attention deficits (Ferreira-Vieira et al. 2016). ChEIs such as tacrine, donepezil, rivastigmine, galantamine is, therefore, the currently approved drugs (Ray and Lahiri 2009; Nazem et al. 2015). However, they have failed to delay or prevent the progression of the disease; provided mild symptomatic benefits (Yiannopoulou et al. 2019).

Recent studies have uncovered the role of neuroinflammation and aberrant gliosis in AD (Imbimbo et al. 2010). Microglia, the primary immune cells of the central nervous system (CNS), can bind Aβ and trigger sustained low-grade neuroinflammation that contributed to altered neuronal integrity (Liddelow et al. 2017) and enhanced Aβ production (Hu et al. 1998; Nhan et al. 2015; Zhu et al. 2018).

Remarkably, neurogenesis occurs until the end of life in cognitively healthy people but declines significantly as AD pathology takes hold (Moreno-Jiménez et al. 2019). Impaired hippocampal neurogenesis is considered one of the major players in cognitive/memory dysfunctions in AD (Hollands et al. 2016). Nestin, an intermediate filament protein and a marker of multipotent neural stem cells (NSCs) (Goldman 2006), has induced neurogenesis in nigral dopaminergic neurons in the mouse model of Parkinson's disease (PD) (Albright et al. 2016). However, its implication in AD is still controversial (Yu et al. 2018; Li et al. 2019). Therapeutic approaches targeting inflammation could manipulate AD-associated impaired neurogenesis (Sung et al. 2020).

Numerous observational studies have elucidated NSAIDs' role in reducing or delaying the progression of AD (Spangenberg and Green 2017). Moreover, decreased incidence of AD was reported in patients taking long-term NSAIDs for rheumatoid arthritis (Jenkinson et al. 1989; Kinney et al. 2018; Zhu et al. 2018). NSAIDs increased lysosomal activity, reduced cerebral plaque load, and, therefore, improved spatial learning and memory (Chandra et al. 2018). Ibuprofen and naproxen has prevented the impaired neurogenesis in AICD transgenic mice (Ghosal et al. 2010). Moreover, subsets of NSAIDs can prevent apoptosis through partially depolarizing mitochondria and preventing Ca^{2+} overload (Sanz-Blasco et al. 2008).

Naproxen, a member of non-selective NSAIDs, has been tested for its neuroprotective effect against AD in the elderly (Martin et al. 2008). It has restored memory function and prevented Aβ-mediated inhibition of long-term hippocampal plasticity (LTP) in the AD transgenic mice model (Kotilinek et al. 2008).

We aimed to study the hypothesis that naproxen, when combined with rivastigmine, could have an additive neuroprotective effect in the AD rat model, which could be related to modifications in neuronal turnover, apoptosis, and neurogenesis. Of all Alzheimer's models, AlCl3-induced brain pathology is more applicable to the sporadic form, the more prevalent type accounting for 95% of all cases (Li et al. 2016). Naproxen potential mitigating effect was investigated through evaluating rats' behavior, hippocampal AChE activity, hippocampal and cerebellar histopathological changes besides investigating neurogenesis and apoptosis markers expression in rivastigmine-treated AlCl3 Alzheimer's rat model.

2. Materials And Methods

2.1. Animals and Experimental design

All the animal procedures were conducted with approval from the institutional Animal Care, and Use Committee of the Faculty of Medicine, Assiut University (IRB no:17100383) and could be given by request. They were carried out following the internationally accepted principles for laboratory animal use and care the US guidelines (NIH publication #85 – 23, revised in 1985). Every effort has been made to reduce the number of animals used and their suffering. Adult male Albino Wistar rats weighing 180–220 g were purchased from the animal house of the Faculty of Medicine, Assiut University. They were housed in stainless steel cages in a well-ventilated room under a 12-h light/dark cycle. Rats had access to water and food *ad libitum*. Rats were randomly divided into five groups (n = 10):

Group 1; Cont group received daily saline injections intraperitoneal (IP) for 60 days.

Group 2; Al group received $AlCl_3$ hexahydrate (qualikemes India), dissolved in saline, daily IP at a dose of 80 mg/Kg per injection for 60 days (Abdel-Aal et al. 2011).

Group 3; RIVA group received $AlCl_3$ and rivastigmine (reference standard drug) (Beijing Mesochem Technology co., Ltd.), dissolved in sterile water, daily IP at a dose of 1 mg/kg per injection for six weeks starting two weeks before $AlCl_3$ administration (Ismail et al. 2013; Zhou et al. 2016).

Group 4; Napro group received $AlCl_3$ and naproxen (Pharm Bio-Tech co., Ltd.), dissolved in saline, daily at a dose of 20 mg/kg per injection IP for two weeks concurrently with $AlCl_3$ (Teng et al. 2011; Sil et al. 2014).

Group 5; RIVA + Napro group received $AlCl_3$, rivastigmine (1 mg/kg for six weeks starting two weeks before $AlCl_3$ administration), and naproxen (20 mg/kg for two weeks concurrently with $AlCl_3$).

2.2. Behavioral studies

A battery of behavioral paradigms was performed in the following sequence; NOR and PA tests.

2.2.1. Novel Object Recognition (NOR) test

The test aims to assess the exploratory behavior, memory, and object recognition based on the intrinsic affinity of rodents to privilege exploring novel objects over familiar ones. The test was carried out in a room illuminated by a ceiling-oriented halogen lamp that gives a uniform dim light. NOR test apparatus is a square stainless steel open field box (60 x 60 x 40 cm) with black walls and floor. NOR test objects were distinct in shape and color and made of heavily painted wood that rats could not displace. They were about 15 cm in height. The test box and objects were cleaned with 70% ethyl alcohol between trials to exclude behavioral tasks associated with olfactory cues. Rats were placed in the experimental room for at least 30 min before testing. They were habituated to the empty test box for 10 min per day for two

consecutive days. A learning session was conducted by placing each rat in the test box with two identical objects for the amount of time necessary to spend a total of 15 sec exploring these two objects to exclude the possibility of random preference. Exploration was considered when, within a cut-off period of 4 min, the rat was touching, looking at, or sniffing with its head within 2 cm of the object. Rats exploring both objects for less than 15 sec were excluded from the experiment. Following the learning phase, three testing sessions were conducted after a retention interval of 5 min, 2 hrs, and 24 hrs to assess short, intermediate, and long-term memories, respectively. Rats were allowed 3 min exploration of one of the objects previously seen during the learning phase and another novel object. Rats with a low level of object exploration, time spent exploring novel and familiar objects < 5 sec, were excluded. As the standard calculation for NOR, memory performance was measured by measuring the recognition index (RI), (time spent exploring the novel object) / (total time spent exploring both objects)*100 (Antunes and Biala 2012).

2.2.2. Passive avoidance (PA) test

The PA test is fear-motivated carried out in rodent models of CNS disorders to assess learning and memory. It is based on the interaction between aversive stimulation, mild foot shock, and a particular environmental situation. The apparatus consists of two chambers separated by a wall containing an 8 cm communicating hole. A single chamber was kept illuminated. The test was performed on two consecutive days. On the first day, rats were individually positioned in the illuminated chamber. Once they reached the dark chamber, an electrical shock was applied to their feet via the floor grid. 24 hrs later, rats were placed again in the illuminated chamber, and the interval till entry to the dark one was recorded; step-through latency (Abdel-Aal et al. 2011).

2.3. Acetylcholinesterase (AChE) activity

After carrying out all the behavioral experiments, the animal groups were sacrificed, and their brains were carefully isolated. Hippocampi were weighed, homogenized in phosphate buffer saline (PBS), centrifuged for 5 min at 5000×g, and the supernatant was collected. According to the manufacturer's protocol, the hippocampal AChE activity was determined using ELISA kits (Elabscience Co.). The optical density (OD) was measured at a wavelength of 450 nm using a microplate reader after the enzyme-substrate reaction was terminated. The AChE activity was determined by comparing the OD of the samples to the standard curve.

2.4. Histopathological studies

Rats were anesthetized with thiopental sodium (50 mg/kg) IP (Abdi-Azar and Maleki 2014) at the end of the experiment. Their hearts were exposed and transcardially perfused through the left ventricle with saline till getting clear flow, then finalized with 10% formalin. Hippocampal and cerebellar tissues were dissected out and processed for light microscopy and immunohistochemical staining techniques. Some sections were stained with hematoxylin & eosin (Hx&E) for general histopathological examination (Bancroft and Gamble 2008).

2.5. Immunohistochemistry Studies

The isolated hippocampi and cerebellar tissues were fixed in 10% neutral formalin followed by dehydration, clearing, and paraffin embedding. Paraffin sections were cut and incubated at 4°C overnight with the following primary antibodies; anti-GFAP; Ab-1 (Clone GA-5) mouse monoclonal antibody (1:100) (Thermo Fisher Scientific Co, Fermont, California, USA), rabbit antimouse caspase-3 polyclonal antibody (1:100) (Chongqing Biospes co., Ltd. China), mouse monoclonal anti-nestin antibody (Abcam, ab22035, UK) (1:100). After that, sections were stained with an avidin-biotin-peroxidase system with diaminobenzidine as the chromogen (DAKO (HRP; rabbit/mouse/goat (DAB+) code no. K0679; Dako Cytomation) in line with the instructions for the staining procedure contained in the Dako LSAB + System-HRP. Hematoxylin was used to counterstain the sections after they were washed in distilled water.

For negative control staining, some sections were incubated with PBS instead of the primary antibody. In these parts, no immunoreactivity was found.

2.6. Morphometric Studies

Morphometric studies were conducted using a Java-based open-source image processing package, image J. The required parameters were measured in 3 non-overlapped fields/5 sections/3 rats from each group. The measured parameters in Hx&E-stained sections were; 1) the number of dark cells in CA1 fields of the hippocampi and 2) the number of dark cells in Purkinje cell layers of the cerebellar tissues using X40 lens. The measured parameters in immunostained sections were; 1) the number of caspase-3 (+ ve) immunostained cells in CA1 fields, 2) the number of caspase-3 (+ ve) immunostained cells in Purkinje cell layers, and 3) the number of nestin (+ ve) immunostained cells in cerebellum using X40 lens.

2.7. Electron Microscopy Studies

The isolated hippocampi and cerebellar tissues were dissected out with the aid of a dissecting microscope, fixed in glutaraldehyde, and processed for transmission electron microscopy. Semi-thin sections (0.5-1 μm) were stained with toluidine blue. Ultrathin sections (500-800A), for the selected areas in semi-thin sections, were contrasted with uranyl acetate and lead citrate, examined with the transmission electron microscope (TEM) JEOL (JEM-100 CXII, Tokyo, Japan), and photographed at 80 kV in Assiut University-Electron Microscope Unit.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by multiple t-tests, one-way and two-way repeated-measures ANOVA, followed by post hoc Tukey's test when appropriate. All statistical tests were directed using GraphPad Prism 7 software. The difference among groups was considered significant for $p < 0.05$.

3. Results

3.1 behavioral outcomes

3.1.1 Novel Object Recognition (NOR) test

The time is taken to explore a novel object at 5 min, 2 hrs, and 24 hrs was substantially longer than that of the familiar one in the Cont group. Poor memory performance was observed in the Al group, detected as lack of preference for a novel object relative to the Cont group ($p < 0.01$, 5 min; 2 hrs; $p < 0.05$, 24 hrs) (Fig. 1). The RIVA group spent considerably more time investigating the novel object compared to the Al group ($p < 0.05$, 5 min; 2 hrs; 24 hrs) (Fig. 1). Treatment with naproxen substantially improved the time spent investigating the novel object at the specified time points ($p < 0.01$, 5 min; 2 hrs; $p < 0.05$, 24 hrs) (Fig. 1), reflecting its ability to rescue memory deficit in the AD rat model. The RIVA + Napro group did not show any additive effect than the RIVA group (Fig. 1).

3.1.2 Passive avoidance (PA) test

Al group showed a significant decrease in step-through latency than the Cont group ($p < 0.01$), signifying marked cognitive deficits. RIVA and Napro groups exhibited a significant increase in step-through latency than the Al group ($p < 0.05$, $p < 0.01$), reflecting improvement in learning and memory tasks. There were no significant differences in retention latency time between RIVA + Napro and RIVA groups (Fig. 2).

3.2 Acetylcholinesterase (AChE) activity

AChE activity was evaluated in the hippocampi of the studied groups. Hippocampal AChE activity was significantly enhanced, an indication of cholinergic impairment, in the Al group when compared to the Cont group ($p < 0.01$) (Fig. 3). RIVA and Napro groups dramatically reduced hippocampal AChE activity compared to the Al group ($p < 0.05$, $p < 0.01$) (Fig. 3). Compared to the RIVA group, the RIVA + Napro group showed a non-significant change in hippocampal AChE activity (Fig. 3).

3.3 Histopathological studies

Examination of Hx&E stained sections of the Cont group brain revealed the normal structure of the rat hippocampus. It was formed of an outer blade (Ob) and an inner blade (Ib) of the dentate gyrus (DG), CA1, CA2, and CA3 fields of Ammon's horn (AH) (Fig. 4a). Cont CA1 field showed stratum oriens (SO), stratum pyramidal (SP), and stratum radiatum (SR) that extended from the alveus to the hippocampal fissure (Fig. 4a). The pyramidal neurons (P), the principal cell type in the CA1 field, were characterized by their triangular perikarya, medium-sized round vesicular nuclei (N), and basophilic cytoplasm. Their processes extended to SR (Fig. 4b). The Al group showed multiple intensely stained irregular pyramidal neurons surrounded by empty spaces (Fig. 4c). Most pyramidal neurons (P) appeared normal with round vesicular nuclei (N), yet few cells still seemed irregular and deeply stained in the RIVA group (Figs. 4d, 4e). Some pyramidal neurons (P) seemed regularly shaped with round vesicular nuclei in the RIVA + Napro group (Fig. 4f). Our morphometric results revealed a significant increase in the number of dark cells ($p < 0.0001$) in the Al group compared to the Cont group; however, a significant decrease in their numbers was observed in the treated groups (Fig. 4g). The RIVA + Napro combination showed a significant decrease in the number of dark cells compared to the RIVA group ($p < 0.001$) (Fig. 4g). Hx&E stained sections of the cerebellar cortex were examined. Cont group cerebellar cortex was composed of an outer molecular layer

(OL), a middle Purkinje cell layer (PL), and an inner granule cell layer (GL) (Fig. 5a). The granule cell layer was packed with granule cells with round nuclei and separated by small acidophilic areas, the cerebellar glomeruli (Fig. 5a). The Purkinje cell layer consisted of one layer of large flask-shaped Purkinje cells with a large central vesicular nucleus (Fig. 5a). The Purkinje cell layer was the most affected as most cells were shrunken deeply stained, leaving empty spaces in the Al group (Fig. 5b). Most Purkinje cells attained their regular flask-shaped appearance with large vesicular nuclei, while few cells were still irregular-shaped and deeply stained in RIVA, Napro, and RIVA + Napro groups (Figs. 5c-5e). Treated groups displayed a significant decrease in the number of dark-stained Purkinje cells ($p < 0.0001$) compared to the Al group (Fig. 5f). Compared to the RIVA group, the RIVA + Napro group had no additive effect (Fig. 5f).

3.4 Immunohistochemical Studies

3.4.1 GFAP expression (hippocampus; CA1 field)

High expression of GFAP is associated with astrocyte activation, which can contribute to brain injury. GFAP immunostained sections of the Cont group showed few immunostained short processed glial cells (Fig. 6a). On the contrary, a marked increase in long processed GFAP immunostained cells in all layers of CA1 fields was displayed in Al, RIVA, Napro, and RIVA + Napro groups (Figs. 6b-6e).

3.4.2 Active caspase-3 expression (hippocampus; CA1 field & cerebellar cortex; Purkinje cell layer)

 # Hippocampus; CA1 field

Caspases-3 is one of the most important proteases involved in the caspase cascade that leads to apoptosis. Cont group revealed few caspase-3 (+ ve) immunostained cells (Fig. 7a). There was a significant increase in caspase-3 (+ ve) immunostained cells in the Al group (Fig. 7b). Caspases-3 (+ ve) immunostained cells were significantly reduced in the RIVA, Napro, and RIVA + Napro classes (Figs. 7c-7e). In the Al group, morphometric findings showed a substantial increase in caspase-3 (+ ve) immunostained cells ($p < 0.0001$). In contrast, treated groups showed a significant reduction in caspase-3 (+ ve) immunostained cells ($p < 0.0001$) compared to the Al group. Caspase-3 expression was significantly lower in the RIVA + Napro group relative to the RIVA group ($p < 0.05$) (Fig. 7f), indicating that naproxen provided an extra neuroprotective effect when concurrently administered with rivastigmine compared to rivastigmine only therapy.

 # Cerebellar cortex; Purkinje cell layer

Cont group immunostained sections showed few caspase-3 (+ ve) immunostained cells (Fig. 7g). Al group, however, revealed increased caspase-3 (+ ve) immunostained cells (Fig. 7h). On the contrary, a marked reduction in caspase-3 (+ ve) immunostained cells were detected in RIVA, Napro, and RIVA + Napro groups (Figs. 7i-7k). Statistical analysis revealed a significant increase in caspase-3 (+ ve)

immunostained cells in the Al group ($p < 0.0001$) compared to the Cont group. In contrast, a significant decrease in caspase-3 (+ ve) immunostained cells was noticed in treated groups ($p < 0.0001$) compared to the Al group (Fig. 7l). Yet, the combination therapy did not provide extra benefit to rivastigmine mono-therapy.

3.4.3 Nestin expression (Cerebellar cortex; granule cell layer)

Decreased nestin (+ ve) immunostained cells were observed in the Al group (Fig. 8b) relative to the Cont group (Fig. 8a), indicating impaired neurogenesis in the granule cell layer of the cerebellar cortex. RIVA, Napro, and RIVA + Napro groups showed increased nestin (+ ve) immunostained cells (Figs. 8c-8e) compared to the Al group indicating enhanced neurogenesis. Statistically, a significant reduction in nestin (+ ve) immunostained cells were observed in the Al group ($p < 0.0001$) compared to the Cont group. Napro group displayed a non-significant enhancement in nestin expression compared to the Al group (Fig. 8f). The RIVA and RIVA + Napro groups showed a substantial increase in nestin (+ ve) immunostained cells relative to the Al group ($p < 0.01$, $p < 0.0001$) (Fig. 8f). The combination, however, exerted an additive effect relative to rivastigmine alone ($p < 0.01$) (Fig. 8f).

3.5 Electron microscopy studies

 # Hippocampus (CA1 field; pyramidal cells & astrocytes)

The pyramidal neurons of the Cont group revealed normal fine structure (Fig. 9a); within abundant cytoplasm that contained mitochondria, rough endoplasmic reticulum (rER), and large ribosomes, they had large round nuclei with finely dispersed chromatin (Figs. 9a, 9b). The Cont astrocyte showed a regular oval euchromatic nucleus and minimal electrolucent cytoplasm enclosing mitochondria and ribosomes (Fig. 9h). Al group, most pyramidal neurons tended to have rarified cytoplasm (Fig. 9c), while others looked degenerated electron dense with ill-defined nuclei (Fig. 9d). Astrocytes had electrolucent swollen rarified cytoplasm (Fig. 9i). The RIVA group showed regenerated pyramidal neurons with electrolucent cytoplasm and large round euchromatic nuclei (Fig. 9e). Some cells were electrolucent with euchromatic nuclei in the Napro group, while others looked degenerated and electron-dense (Fig. 9f). Some pyramidal neurons appeared regenerated with electrolucent cytoplasm and euchromatic nuclei in RIVA + Napro (Fig. 9g); nevertheless, astrocytes still attained swollen rarified cytoplasm (Fig. 9j).

 # Cerebellar cortex (granule cell layer & Purkinje cells)

Cont group granule cells were seen in clusters in the granule cell layer (Fig. 10a). Their nuclei appeared oval or rounded, surrounded by minimal electrolucent cytoplasm that enclosed few mitochondria and ribosomes (Fig. 10a). Purkinje cells were large-sized with large euchromatic nuclei surrounded by voluminous cytoplasm that enclosed abundant mitochondria, rER, and ribosomes (Fig. 10f). Al granule cells tended to have heterochromatic nuclei and electron-dense rarified cytoplasm (Fig. 10b). Purkinje

cells perikaryon appeared degenerated electron dense with ill-defined nuclei and organelles (Fig. 10g), while others seemed electron-dense and surrounded by swollen astrocytic processes (Fig. 10h). Normal fine structure of granule cells was attained in the treated groups (Figs. 10c-10e). Purkinje cells appeared less electron-dense with a well-defined nucleus and abundant cytoplasm rich in organelles in the RIVA group (Fig. 10i). In contrast, some Purkinje cells in the Napro group were still electron-dense with ill-defined nuclei and organelles (Fig. 10j). Purkinje cells had abundant electrolucent cytoplasm with large euchromatic nuclei surrounded by swollen astrocytic cytoplasm in the RIVA + Napro group (Fig. 10k).

4. Discussion

Our research was the first to show that naproxen could have an additive neuroprotective effect in the AlCl3-induced Alzheimer's rat model when given simultaneously with rivastigmine, the standard anti-Alzheimer medication, by altering neuronal turnover, enhancing neurogenesis, and suppressing apoptosis. Naproxen has mitigated AlCl3-mediated neurocognitive deficit as evidenced by improved memory performance in the NOR test and reduced step-through latency in the PA test, besides ameliorating cholinergic deficits through decreased hippocampal AChE activity. Compared to rivastigmine alone, naproxen had no additional ameliorative influence on AD-related neurocognitive deficits when given synchronously with rivastigmine.

AD is an irreversible debilitating age-related neurologic disorder affecting over 47 million people worldwide. It is one of the most disappointing medical concerns that greatly distress societies and their economy (Colucci et al. 2014; Liu et al. 2014), especially as the population ages globally (Caselli et al. 2006; Arshavsky 2010). The disease hallmark is an insidious progressive intellectual decline that commonly involves the memory of recent facts, executive functions, and spatial orientation (Benedikz et al. 2009). Speech, mobility, and neuropsychiatric problems are among the patients' sufferings (Sun et al. 2013). Therefore, timely diagnosis plus achieving effective therapeutic approaches are mandatory.

AD is classically characterized by β-amyloid plaque deposits of Aβ peptides, activated microglia, reactive astrocytes, and NFTs (Beach et al. 1989; Iqbal and Grundke-Iqbal 2008; Weller and Budson 2018). Aβ brain aggregation, mediated through imbalance between their production and clearance, provokes subsequent pathological events as oxidative stress and neuroinflammation that eventually lead to neuronal loss (Mawuenyega et al. 2010; Mucke and Selkoe 2012; Liu et al. 2016). Activated microglia and reactive astrocytes, tightly associated with amyloid plaques (Olabarria et al. 2010), augment the local inflammatory response (Nagele et al. 2003; Rodríguez et al. 2009).

Memory and cognitive decline are directly correlated with cerebral cholinergic neuron degeneration within the basal forebrain (Whitehouse et al. 1981, 1982). Decreased acetylcholine (ACh) release, impaired choline acetyltransferase action, or increased activity of AChE further aggravate Ach scarceness in AD (Fishman et al. 1986; Hammond and Brimijoin 1988; Rodríguez-Puertas et al. 1994). NMDA (N-methyl-D-aspartate) receptors overactivation is fundamental for AD progression (Dingledine et al. 1999). ChEIs as

tacrine, donepezil, rivastigmine, and galantamine (Ray and Lahiri 2009; Nazem et al. 2015) for mild to moderate circumstances and NMDA antagonists as memantine for moderate to severe cases are the currently approved therapy. They can only provide symptomatic relief, despite being partly effective in the early stages (Citron 2010; Alteri and Guizzaro 2018). The standard anti-Alzheime drug, rivastigmine (Birks and Evans 2015; Kandiah et al. 2017), can enhance intellectual functioning through raising synaptic ACh levels mainly within the hippocampus and neocortex (Gothwal et al. 2019).

A potential association between AD incidence and aluminum (Al) content in drinking water has been indicated (McLachlan et al. 1996; Altmann et al. 1999; Gauthier et al. 2000; Peder Flaten 2001; Exley and Esiri 2006). An elevated concentration of Al was detected in AD patients' brains (Crapper et al. 1973). Al industry personnel serving in miners, foundry, and welders have displayed impaired cognitive functions (Rifat et al. 1990; Polizzi et al. 2002; Giorgianni et al. 2003). Excess Al intake induces amyloid-beta precursor protein (APP) overexpression, amyloid deposition (Castorina et al. 2010), apoptosis activation besides degenerative neuronal changes (Crapper et al. 1973; Zatta et al. 2003; Kawahara and Kato-Negishi 2011).

Divergent aspects of AD have been studied through numerous categories of animal models. Rats are considered the favored species (Benedikz et al. 2009) as they are brainy, quick learners besides their similarities in physiological processes (Benedikz et al. 2009) and pathological alterations to human beings (Herrera et al. 1999; Reid et al. 2001; Hörsten et al. 2003; Loeffler 2004). We, therefore, decided to study the AlCl3 induced Alzheimer rat model as it is the most relevant to the sporadic AD pathology (Evrard et al. 1998; Li et al. 2016). Chronic AlCl3 administration enhances Al CNS access through the blood-brain barrier (BBB) via special high-affinity transferrin receptors (Roskams and Connor 1990). Al accumulates in all rat brain areas (Abubakar et al. 2004; Sakamoto et al. 2004; Kaur and Gill 2006), although the cerebral cortex, the hippocampus, and the cerebellum are the structures most commonly implicated in the lesions initiated by Al intoxication (Slanina et al. 1984; Misawa and Shigeta 1992). Our study showed significant neuronal degeneration presented as marked shrunken dark basophilic neurons with infiltration of glial cells within the CA1 pyramidal layer in the Al group, consistent with previous studies (Junior et al. 2013; Said and Rabo 2017; Bazzari et al. 2019). A significant increase in the deeply stained neurons within the Purkinje cell layer associated with electron-dense cytoplasm, ill-defined nuclei, and organelles within the Purkinje and granular cell layers were displayed within Al group cerebellar tissues. Our findings are in line with other studies (Bhalla and Dhawan 2009; Bondy 2016) that revealed disorganization in the architecture of the Purkinje cell layer with a loss of Purkinje cells of cerebellar cortex in the AD rat model. RIVA group revealed the more or less normal histological structure of the hippocampus CA1 field and cerebellum Purkinje cell layer with a substantial reduction in the number of dark or deeply stained cells that is in line with other research (Mahdy et al. 2012), confirming the mitigating impact of rivastigmine on AlCl3-induced neurotoxicity.

AlCl3 AD rat model displayed a biphasic response of AChE activity; an increase in AChE activity in short-term administration followed by pronounced decay of the enzyme activity on long-term (Kumar 1998; Kaizer et al. 2008). Long-term response ensues by a slow accumulation of Al with the formation of a

complex with the anionic site of the AChE enzyme (Kumar 1998; Kaizer et al. 2008; Nampoothiri et al. 2015). In agreement with other studies (Zheng et al. 2009; Said and Rabo 2017), our findings revealed a significant increase in hippocampal AChE activity in the Al group indicating short phase response. Following previous research (Onor et al. 2007; Nampoothiri et al. 2015, 2017), our study showed that rivastigmine inhibited AlCl3-enhanced hippocampal AChE activity, which partially enhanced cholinergic neurotransmission. In advanced cases, however, rivastigmine exerts its action by inhibiting BuChE activity (Greig et al. 2001; Giacobini et al. 2002).

AlCl3-treated animals have demonstrated major memory and cognitive deficits (Ikram et al. 2020; Mustafa 2020). They showed increased retention latency and decreased RI percentage (Jangra et al. 2015), indicating decreased short, intermediate, and long-term memory. Intracerebral administration of AlCl3 triggered learning deficits in rabbits as well (Rabe et al. 1982). The NOR test revealed that the Al group had a week exploratory preference compared to the Cont group and degradation in spatial and retention memory as measured by PA test, which is consistent with previous literature (Lakshmi et al. 2015).

Rivastigmine attenuated AlCl3-induced spatial and retention memory deficit (Mehrabadi et al. 2020). Decreased escape latency and increased discrimination index were reported in the streptozotocin rat model of AD treated with rivastigmine (Akhtar et al. 2020). Moreover, rivastigmine exerted positive effects on learning and memory in the chronic d-galactose-induced accelerated aging rodent model (Chogtu et al. 2018). It reversed spatial learning deficits induced by scopolamine as well (Deiana et al. 2009).

Neuroinflammation is acknowledged as a prominent feature in AD pathology (Hensley 2010). Aβ directly activates glial cells, astrocytes and microglia, found near Aβ plaques (Perlmutter et al. 1992; Townsend and Praticò 2005; Schwab and McGeer 2008; Heneka et al. 2015), triggering the release of inflammatory mediators, notably IFNγ, IL-1β, TNF-α, IL-6, TGF-β (Constam et al. 1992; McGeer and McGeer 1995; Hu et al. 1998; Johnstone et al. 1999), free radicals, inducible nitric oxide synthase (iNOS) and nitric oxide (NO) (Hu et al. 1998; Ayasolla et al. 2004; Garção et al. 2006; Furman et al. 2012; Cai et al. 2014). It is believed that reactive astrogliosis, hypertrophy of cell soma, and processes induced by Aβ plays a role in limiting the build-up of Aβ plaques (Olabarria et al. 2010; Hou et al. 2011) through the expression of type III intermediate filament (IF) protein; glial fibrillary acidic protein (GFAP) (Wilhelmsson et al. 2006; Kamphuis et al. 2012). On the contrary, astrogliosis could enhance neurotoxicity with further neuronal death (Scuderi et al. 2014). Its role, therefore, in AD is still controversial. Nevertheless, astrogliosis degree is usually correlated with cognitive decline in AD (Beach and McGeer 1988; Mrak et al. 1996; Kashon et al. 2004).

Our study detected an apparent increase in hippocampal GFAP immunoreactivity in AlCl3-intoxicated rats suggesting severe astrocytic activation induced by Al uptake. Our results are consistent with those of other studies (Guo-ross et al. 1999; Li et al. 2009; Erazi et al. 2010; Justin-Thenmozhi et al. 2018), which detected increased GFAP immunolabelling in the hippocampus and frontal cortex after chronic Al intoxication in rats and rabbits (Yokel and Callaghan 1998) though unchanged GFAP immunoreactivity

was still reported (Platt et al. 2001). Other studies, on the contrary, revealed a decrease in GFAP immunoreactivity, reflecting astrocytes' susceptibility to Al-induced neurotoxicity (Guo-ross et al. 1999; Junior et al. 2013). RIVA group showed enhanced GFAP immunoreactivity indicating persistent astrogliosis, which contradicts other studies (Mohamed et al. 2016) that displayed reduced immunoreactivity by 45–50%. Similarly, rivastigmine has attenuated the memory decline in T2DM-AD model mice by suppressing gliosis (Matsuda and Hisatsune 2017). The diversity of the AD models may lie behind the conflicting outcomes.

Microglia were studied to play dual roles in Aβ pathogenesis (Ard et al. 1996; Grathwohl et al. 2009; Majumdar et al. 2011). Early microglial recruitment enhances Aβ clearance across the BBB via microglia scavenger receptors (Khoury et al. 1996; Paresce et al. 1996; Kunjathoor et al. 2004; Alarcón et al. 2005; Yang et al. 2011). Chronic inflammation, however, mediates microglial dysfunction, which plays a detrimental effect via the release of cytotoxic molecules, enhanced production, and impaired clearance of Aβ (Hickman et al. 2008; Brandenburg et al. 2010; Feng et al. 2011). This is likely mediated by decreased expression of enzymes that degrade Aβ; insulysin, neprilysin, and metallopeptidase 9 matrix (MMP9); and loss of functional and structural integrity BBB (Hickman et al. 2008; Brandenburg et al. 2010; Feng et al. 2011).

A vicious cycle of inflammation has been collectively designed between Aβ accumulation, activated microglia, and microglial inflammatory mediators. Therefore, a therapeutic intervention targeting neuroinflammation, particularly in early disease processes (McGeer et al. 1996; Stewart et al. 1997; Vlad et al. 2008), seems to potentially restrain AD's further progression. NSAID use is associated with a lower risk of developing AD in a range of ethnodemographic populations (Breitner et al. 1995; Stewart et al. 1997; Veld et al. 2001; Landi et al. 2003; Fischer et al. 2008; Szekely et al. 2008; Vlad et al. 2008; Côté et al. 2012) besides various preclinical studies (Lim et al. 2000; Weggen et al. 2001; Yan et al. 2003). Importantly, a greatly reduced risk of AD was noticed in rheumatoid arthritis patients on long-term NSAIDs (Mcgeer et al. 1990). Moreover, chronic therapy improved behavioral deficits in AD rodent models (McGeer and McGeer 2007). NSAIDs acts through inhibiting cyclooxygenase (COX) (Varvel et al. 2009), in particular, COX-1, as it is primarily expressed in microglia (Deardorff and Grossberg 2017), leading to blockade of microglial activation besides altered immune cells infiltration. COX-1 deficient mice exhibit reduced inflammation and neuronal injury levels in response to Aβ (Choi and Bosetti 2009). NSAIDs can act as α-, β-, and γ-secretase modulators, which help lessen amyloid deposition (Kukar and Golde 2008). Furthermore, they can directly interact with the Aβ peptide inhibiting the formation of Aβ oligomers and deposits (Kukar et al. 2008), which consequently attenuate microglial activation, the number of reactive astrocytes, and expression of proinflammatory molecules (Lim et al. 2000; Jantzen et al. 2002; Richardson et al. 2002; Yan et al. 2003; Heneka et al. 2005; Kotilinek et al. 2008). PPAR-gamma agonists as ibuprofen, indomethacin, and naproxen decrease promoter activity of the beta-site APP-cleaving enzyme 1 (BACE1) involved in APP processing as well (Sastre et al. 2006). Therefore, NSAIDs' beneficial effects have been attributed to their ability to reduce Aβ generation, plaque size, and tau phosphorylation (Yoshiyama et al. 2007; Khoury and Luster 2008) with subsequent improvement in behavioral impairments (Lim et al. 2000, 2001; Sung et al. 2004; Kukar et al. 2007).

NSAIDs can prevent the intellectual decline in older adults if started earlier, before age 65 (Hayden et al. 2007). Existing cognitive impairments limit their therapeutic usefulness (Martin et al. 2008). Ibuprofen and naproxen can block the early appearance of neuronal ectopic cell cycle events (CCEs), an early marker for risk of neurodegeneration (Busser et al. 1998; Yang et al. 2001, 2003), however, failed to reverse the current events in older mice (Varvel et al. 2009). Unexpectedly, NSAIDs can accelerate AD pathogenesis in advanced cases (Breitner et al. 2009) possibly, through inhibiting microglia-mediated clearance of Aβ and the compensatory neurogenesis processes.

Naproxen, a common over-the-counter medicine, could help to prevent the progression of AD (Kim et al. 2011) through destabilizing preformed Aβ fibrils, reducing their amounts (Agdeppa et al. 2003; Hirohata et al. 2005) besides antagonizing Aβ aggregation (Cole and Frautschy 2010). Naproxen chronic administration blocked alterations in brain microglia and neuronal cell cycle events in young transgenic animals (Imbimbo et al. 2010). Long-term prophylactic use may reduce the risk of AD by 67% compared to placebo (Vlad et al. 2008; Imbimbo 2009; Imbimbo et al. 2010), though; it offers no therapeutic influence in preexisting AD cases (Gasparini et al. 2004; Imbimbo 2004). Our findings established the ability of naproxen to restore the architecture of pyramidal and Purkinje cells, although some of them were still shrunk, irregular, and deeply stained. Compared to the RIVA group, the RIVA+Napro proved to have an additive neuroprotective effect on the hippocampal pyramidal cells but could not provide extra benefit to the cerebellar Purkinje cells.

In our research, naproxen had a mitigating effect over AlCl3 anticholinergic action through decreased AChE activity. Hence, our findings agree with previous studies (Mostafa et al. 2016) that showed naproxen's ameliorative effect on AD-like behavioral performance through attenuating AChE activity. Our findings confirmed naproxen's ability to alleviate intellectual dysfunction caused by AlCl3 as evidenced by significantly augmenting the time exploring the novel objects, step-through latency, and spatial learning compatible with other studies' results (Jain et al. 2002). Chronic treatment with naproxen significantly improved colchicine-induced cognitive impairment (Kumar et al. 2006). Moreover, naproxen has potential neuroprotective properties against D-serine mediated excitotoxicity implicated in PD, Huntington's disease, multiple sclerosis, and AD (Armagan et al. 2012). Some reports, however, have argued against naproxen anti-Alzheimer role (Lyketsos et al. 2007; Martin et al. 2008; Imbimbo et al. 2010). When compared to the RIVA group, the RIVA+Napro group was unable to provide an additive cognitive improvement.

AD neuronal cell death can be attributed to apoptosis and DNA fragmentation (Chang et al. 2016). Disruption of mitochondrial homeostasis by high levels of reactive oxygen species (ROS) mediates the release of proapoptotic cytokines as cytochrome c (Tyagi et al. 2006; Liu et al. 2015) that bind to apoptotic protease activating factor 1 (Apaf-1) (Skulachev 1998; Ghribi et al. 2002; Al-olayan et al. 2015; Resseguie et al. 2015). This complex activates caspase-3, the major executioner in apoptosis (Lynch et al. 2000; Sjöbeck and Englund 2001; Chang et al. 2016), via proteolytic cleavage (Allan and Clarke 2009; Reubold and Eschenburg 2012) into two subunits which dimerize to form the active enzyme (Rotonda et al. 1996). Aβ can induce neuronal apoptosis via activation of caspase-3 found colocalized within senile

plaques of AD brains (Amelio et al. 2012; Cetin et al. 2013; Chang et al. 2016). Besides, studies have illustrated an apoptogenic role of AChE (Zhang and Greenberg 2012), which revealed the potential value of AChEIs therapeutics in early AD (Toiber et al. 2008).

Our study showed that the protein level of the activated caspase-3 was significantly augmented in AlCl3-intoxicated rats in the hippocampus and cerebellar tissues compared with the Cont group, which is in line with other research (Greilberger et al. 2008; Porsteinsson et al. 2008; Kumar and Kumar 2009; Al-olayan et al. 2015; Alawdi et al. 2017; Justin-Thenmozhi et al. 2018; Attia et al. 2020). AlCl3-induced activated caspase-3 overexpression was significantly attenuated by the concomitant treatment with rivastigmine. This is in line with other study reports that showed an ameliorating effect of rivastigmine over AD-mediated caspase-3 overexpression (Elmegeed et al. 2015; Sachdeva and Chopra 2015). Similarly, activated caspase-3 overexpression caused by AlCl3 was significantly attenuated by concomitant naproxen administration. NSAIDs are competitive multi-caspase inhibitors, but their actions are more pronounced for caspases-4, -5, and -9, with lower activity against caspase-3 and -1 due to variations in the pocket recognition substrates (Smith et al. 2017). Caspase inhibition is recognized as a Cox-independent (Chan 2002) anti-inflammatory mechanism for NSAID drugs with a consequent decrease in cell death and proinflammatory cytokine production (Smith et al. 2017). When naproxen was given with rivastigmine, it had an additive antiapoptotic effect on the hippocampal CA1 region compared to rivastigmine alone. Still, according to our findings, it failed to have an additive antiapoptotic to the cerebellar Purkinje cells. On the contrary, other studies showed the ability of NSAIDs to induce apoptosis which lies behind their chemopreventive impact against cancer of the gastrointestinal tract (gastric or colorectal cancer) (Chan 2002; Jana 2008) lung, breast, and prostate cancers (Chan et al. 2005; Rothwell et al. 2010, 2012; Shebl et al. 2014; Seetha et al. 2020).

Recent studies have suggested the implication of neurogenesis in neurodegenerative disorders (Zhao et al. 2008; Lazarov et al. 2010; Mu and Gage 2011; Marxreiter et al. 2013). Deficits in adult neurogenesis may contribute to tau hyperphosphorylation in new neurons, compromised hippocampal circuitry, and cognitive impairments in AD (Hollands et al. 2017). Therefore, through pharmacological and genetic approaches, induction of neurogenesis can slow down disease progression (Cho et al. 2007). The neurogenesis process has been well acknowledged in two brain regions; the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus DG (Zhao et al. 2008). Nevertheless, neurogenesis has still been specified in other regions of the adult mammalian brain as the neocortex, cerebellum, striatum, amygdala, and hypothalamus (Radad et al. 2017). The cerebellum encloses much of the adult brain's mature neurons (Wingate and Hatten 1999). It has a remarkable feature of being able to regenerate its cells by neurogenesis following damage (Andreotti et al. 2018). Strikingly, after its ablation by irradiation, the cerebellar external granular layer can be reconstituted (Altman et al. 1969) probably through cerebellar nestin-expressing progenitors, residing mostly in the Purkinje cell layer, that can differentiate into granule cell precursors (GCPs) and mature granule neurons (Wojcinski et al. 2017). Our study showed a significant reduction of nestin immunoreactivity in the granule cell layer of the Al group compared to the Cont group indicating impaired cellular proliferation

that is consistent with other studies (Yu et al. 2018; Li et al. 2019; Ibrahim et al. 2021). However, concomitant administration of rivastigmine increased nestin immunoreactivity, presumably, due to compensation for cholinergic deficits (Tayebati et al. 2004). This was consistent with other research findings (Salem et al. 2014) that showed enhanced expression of the brain nestin gene by 65.2% through rivastigmine administration relative to the untreated AD population. Similarly, naproxen administration-induced nestin protein overexpression may represent a therapeutic option to restore adult neurogenesis in AD patients. Previous studies showed the potential effectiveness of one of the non-selective COX inhibitors, indomethacin, to restore adult neurogenesis in PD (Hain et al. 2018). When concurrently given with rivastigmine, naproxen exerted an additive effect in promoting neurogenesis relative to rivastigmine-only therapy. Therefore, it could possibly enhance rivastigmine anti-Alzheimer's activity.

Conclusion

AD is an age-related neurodegenerative disorder manifested by cognitive disability commonly associated with neuropsychiatric disorders. The hippocampus and the cerebellum are the most commonly implicated in Al intoxication. They showed major neuronal degeneration with an increased amount of intensely stained shrunken cells within the pyramidal and Purkinje cell layers. Hippocampal astrogliosis with enhanced GFAP expression was detected as well. Naproxen has mitigated AlCl3-induced neurocognitive impairment as it upgraded memory performance, decreased step-through latency, and improved spatial learning and memory retrieval, most likely through decreased hippocampal AChE activity. Our study was the first to demonstrate the neuroprotective effect of naproxen in the AlCl3 Alzheimer's rat model when given concurrently with rivastigmine, the standard anti-Alzheimer drug, as it attenuated neuropathological changes through modifying neuronal turnover; apoptosis suppression (activated caspase-3 downregulation), and enhancement of neurogenesis (nestin upregulation). However, compared to rivastigmine-only therapy, naproxen concurrently administered with rivastigmine had no additional ameliorative effect on intellectual alterations in the AD rat model. Further research is still needed to determine whether naproxen given before clinical dementia or over a longer period will help delay AD development when given in combination with rivastigmine.

Declarations

Ethics approval:

All the animal procedures were conducted with approval from the institutional Animal Care, and Use Committee of the Faculty of Medicine, Assiut University (IRB no:17100383) and could be given by request. They were carried out following the internationally accepted principles for laboratory animal use and care the US guidelines (NIH publication #85-23, revised in 1985).

Consent for publication:

Not applicable.

Availability of data and materials:

Not applicable.

Competing interests:

All the authors declare that there are no competing financial interests concerning the work.

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Authors' contributions:

The studies were conceived and planned by Raafat A. Abdel-Aal and Lobna A. Abdelzaher. The experiments were carried out by Lobna A. Abdelzaher, Reham G. Elsaady, and Ola A. Hussein. The data was analysed by Lobna A. Abdelzaher, Reham G. Elsaady, and Ola A. Hussein. Lobna A. Abdelzaher wrote the manuscript. The manuscript was revised by Raafat A. Abdel-Aal, Reham G. Elsaady, Lobna A. Abdelzaher, and Ola A. Hussein. The final manuscript was read and accepted by all contributors.
All authors read and approved the final manuscript.

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Figures

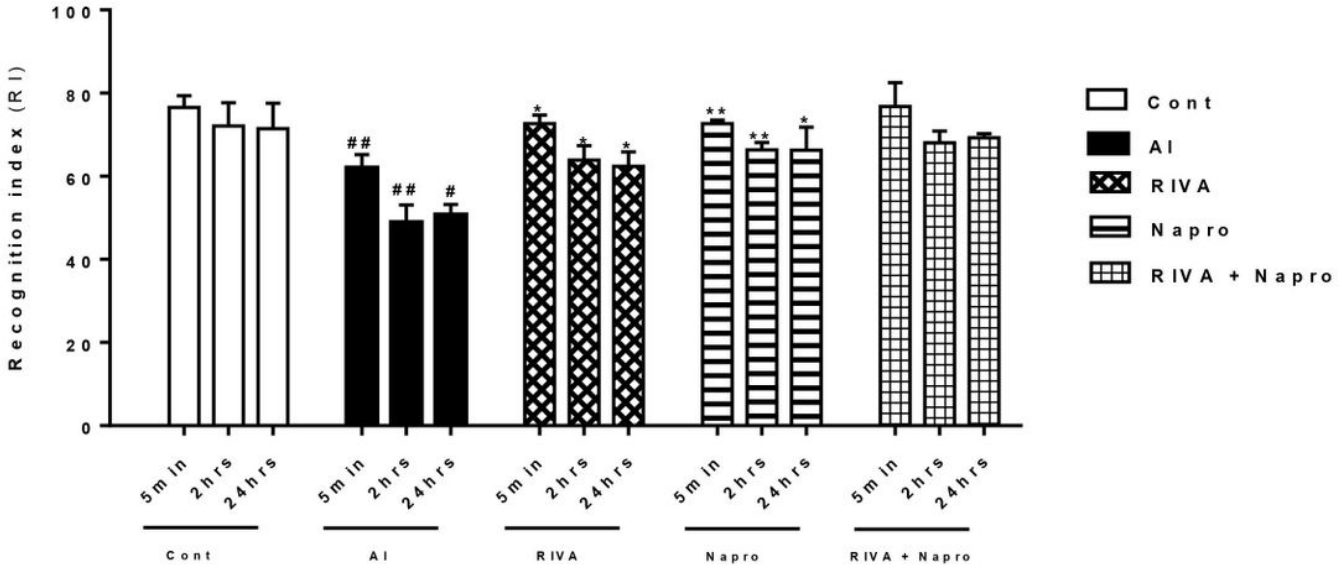

Figure 1

Effect of rivastigmine, naproxen, and their combination on recognition index (RI) of novel object recognition (NOR) test in AlCl3 induced Alzheimer's in rats. The data are expressed as mean ± SEM. #p, *p < 0.05, ##p, **p < 0.01. #, ##: a significant difference from the Cont group. *, **: a significant difference from the Al group.

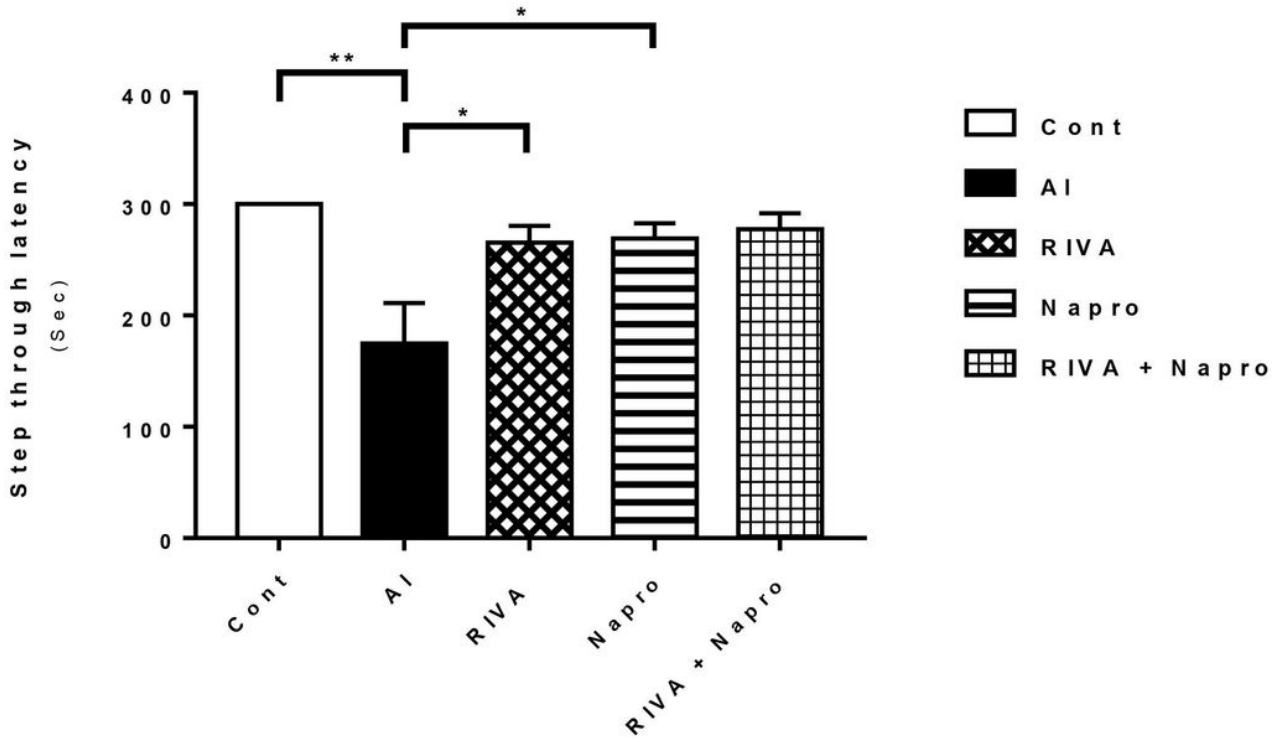

Figure 2

Effect of rivastigmine, naproxen, and their combination on step-through latency of passive avoidance (PA) test in AlCl3 induced Alzheimer's in rats. The data are expressed as mean ± SEM. *p < 0.05, **p < 0.01.

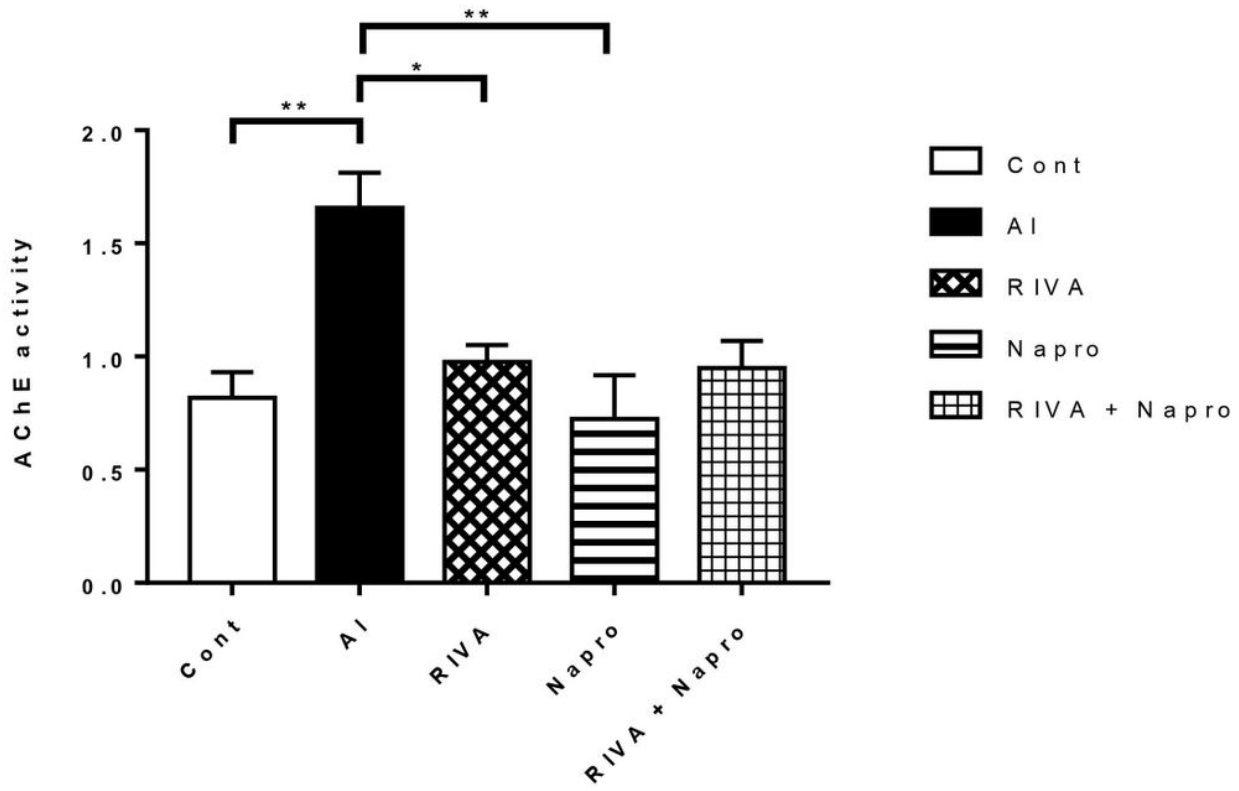

Figure 3

Effect of rivastigmine, naproxen, and their combination on hippocampal acetylcholinesterase (AChE) activity in AlCl3 rat model of Alzheimer disease. The data are expressed as mean ± SEM. *p < 0.05, **p < 0.01.

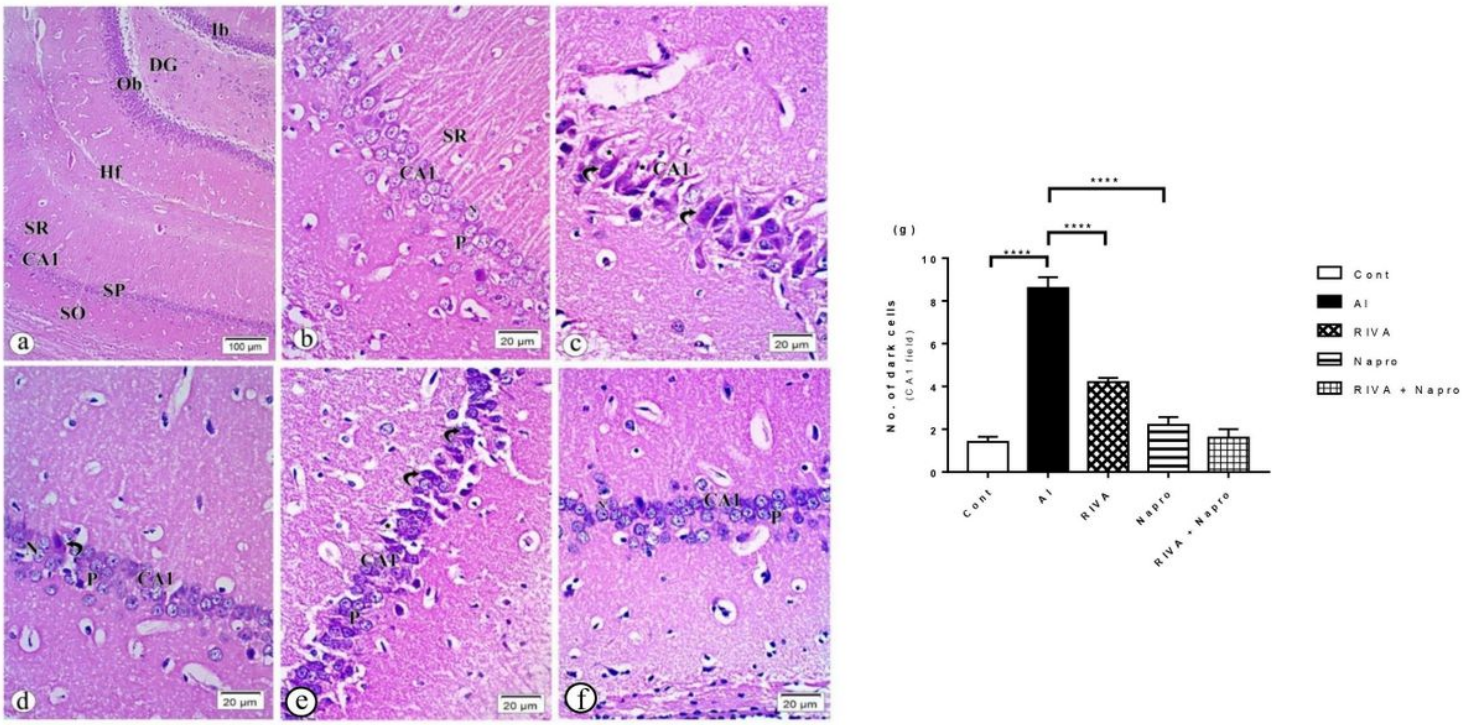

Figure 4

Photomicrographs of Hx&E stained sections of rat hippocampus (CA1 field). a) Cont hippocampus; inner blade (Ib) and outer blade (Ob) of the dentate gyrus (DG), CA1 of Ammon's horn from alveus to hippocampal fissure (Hf), stratum oriens (SO), stratum pyramidal (SP), and stratum radiatum (SR) x100. b) Cont CA1; medium-sized pyramidal cells (P), round vesicular nuclei (N), and their processes extending to stratum radiatum (SR) x400. c) Al CA1; many dark shrunken pyramidal cells (curved arrows) surrounded by empty spaces (*) x400. d) RIVA CA1; most pyramidal cells (P) have round vesicular nuclei (N), few of them are still dark (curved arrows) x400. e) Napro CA1; pyramidal cells with round vesicular nuclei (P) while some of them are still shrunken and deeply stained (curved arrows) x400. f) RIVA+Napro CA1; most pyramidal cells (P) are well arranged and have round vesicular nuclei (N) x400. g) Statistical analysis of the number of dark cells in CA1 fields. The data are expressed as mean ± SEM. ***p < 0.001, ****p < 0.0001.

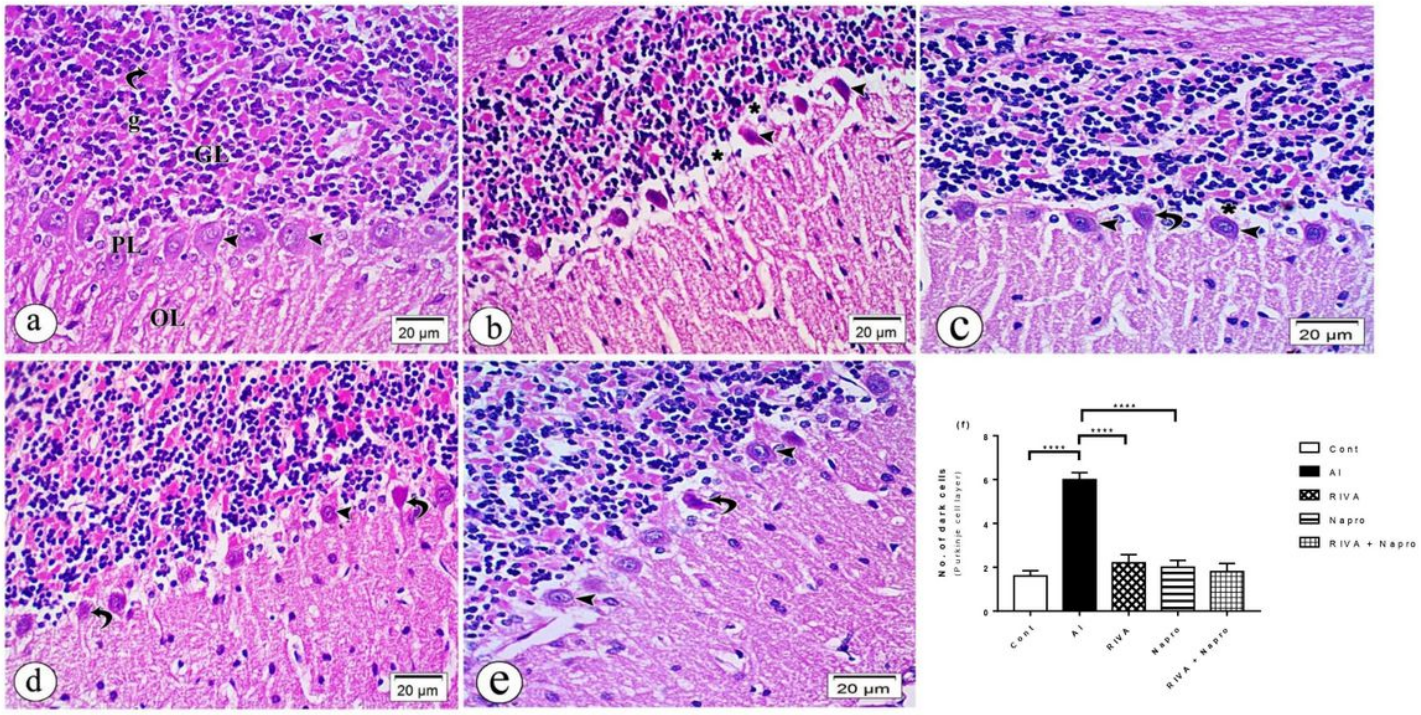

Figure 5

Photomicrographs of Hx&E stained sections of rat cerebellum (Purkinje cell layer). a) Cont cerebellar cortex; outer molecular layer (OL), middle Purkinje cell layer (PL), and an inner granule cell layer (GL). Purkinje cells are large flask-shaped (arrowheads) with large vesicular nuclei. Granule cells (g) in clusters and separated glomeruli (curved arrows) x400. b) Al Purkinje cell layer; most of the Purkinje cells (arrowheads) are shrunken, deeply stained leaving empty spaces (*). c) RIVA Purkinje cell layer; Purkinje cells (arrowheads), few deeply stained cells are seen, empty spaces (*) x400. d) Napro Purkinje cell layer; some flask-shaped Purkinje cells (arrowheads), some irregular shrunken deeply stained cells (curved arrows). e) RIVA+Napro Purkinje cell layer; multiple flask-shaped Purkinje cells with vesicular nuclei (arrowheads), few are irregular deeply stained (curved arrows) x400. f) Statistical analysis of the number of dark cells in the Purkinje cell layer. The data are expressed as mean ± SEM. ****p < 0.0001.

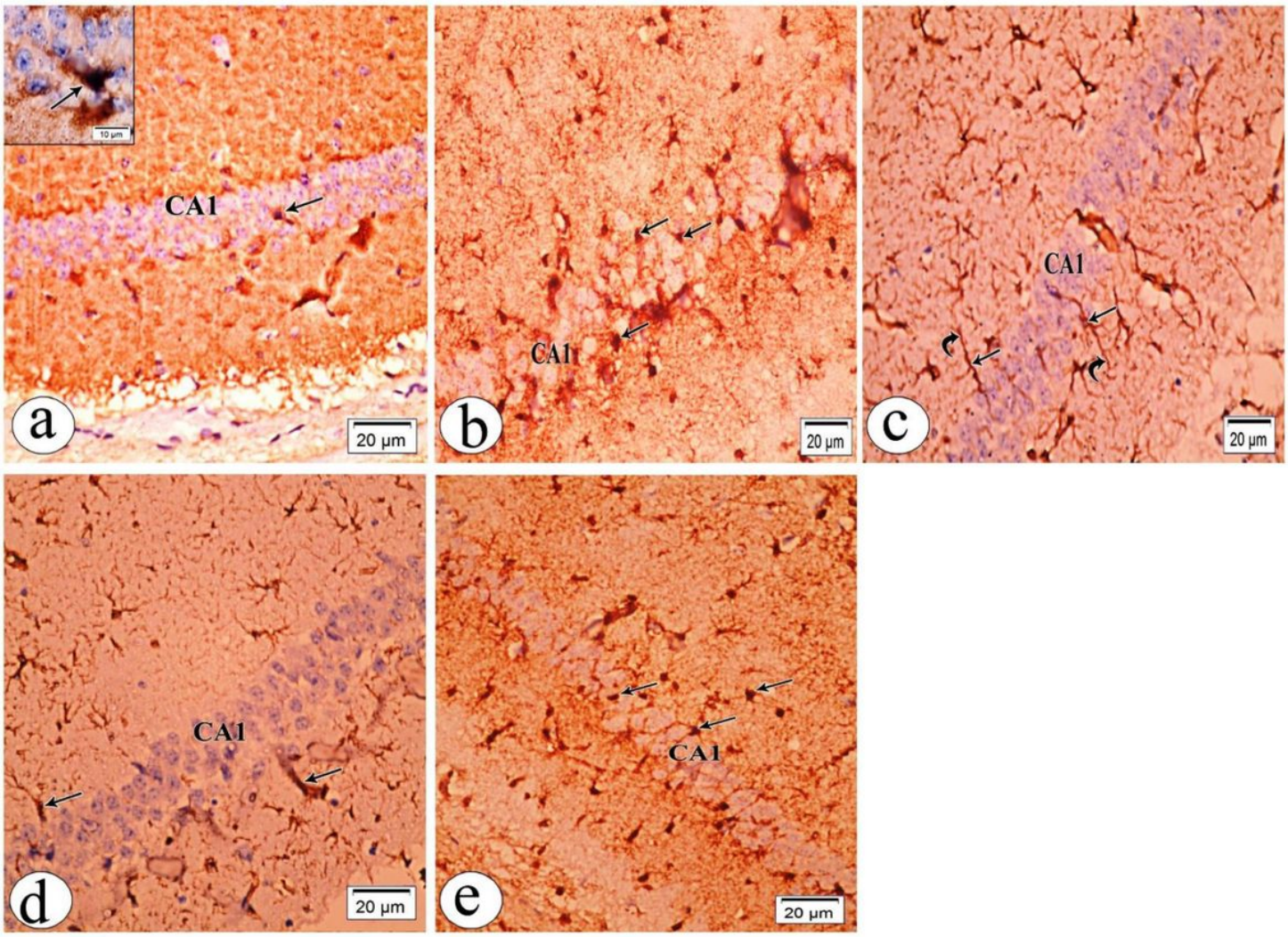

Figure 6

Photomicrographs of GFAP immunostained sections of rat hippocampus (CA1 field). a) Cont CA1; few immunostained cells (↑) with short processes x400, Inset: higher magnification showing glial cells with short processes (↑). b) Al CA1; multiple GFAP immunostained cells (↑) with long processes x400. c) RIVA CA1; multiple GFAP immunostained cells (↑). d) Napro CA1; multiple GFAP immunostained cells (↑) with long processes x400. e) RIVA+Napro CA1; multiple GFAP immunostained cells (↑) x400.

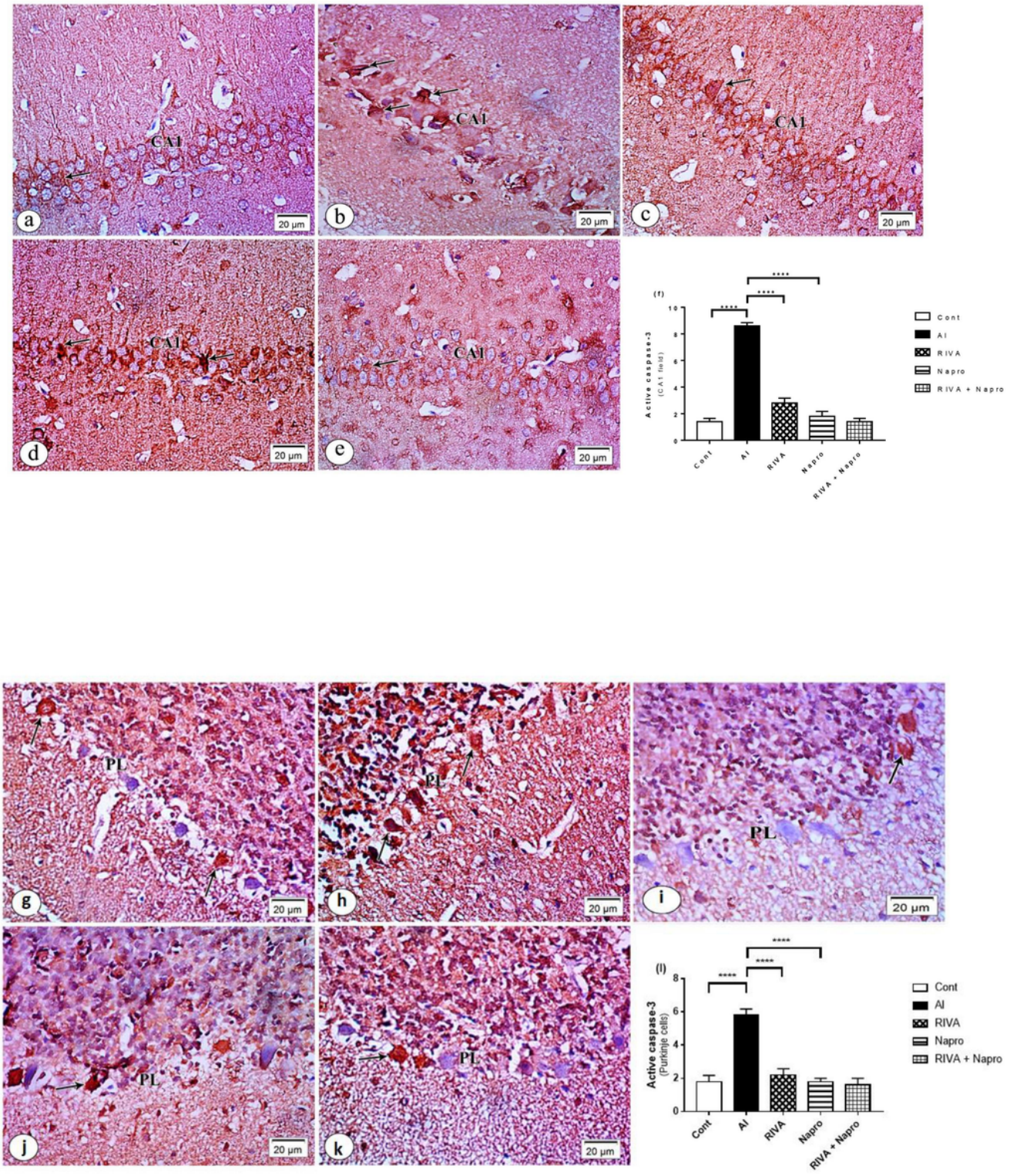

Figure 7

Photomicrographs of active caspase-3 immunostained sections of rat hippocampus (CA1 field) and cerebellum (Purkinje cell layer). a) Cont CA1; few caspase-3 immunostained cells (↑) x400. b) AI CA1; multiple caspase-3 immunostained cells (↑) x400. c) RIVA CA1; few caspase-3 immunostained cells (↑) x400. d) Napro CA1; few caspase-3 immunostained cells (↑) x400. e) RIVA+Napro CA1; few caspase-3 immunostained cells (↑) x400. f) Statistical analysis of the number of caspase-3 immunostained cells in

CA1 fields. The data are expressed as mean ± SEM. ****p < 0.0001. g) Cont Purkinje cell layer; few caspase-3 immunostained cells (↑). h) Al Purkinje cell layer; multiple caspase-3 immunostained cells (↑). i) RIVA Purkinje cell layer; few caspase-3 immunostained cells (↑). j) Napro Purkinje cell layer; few caspase-3 immunostained cells (↑). k) RIVA+Napro Purkinje cell layer; few caspase-3 immunostained cells (↑). l) Statistical analysis of the number of caspase-3 immunostained cells in Purkinje cell layer. The data are expressed as mean ± SEM. ****p < 0.0001.

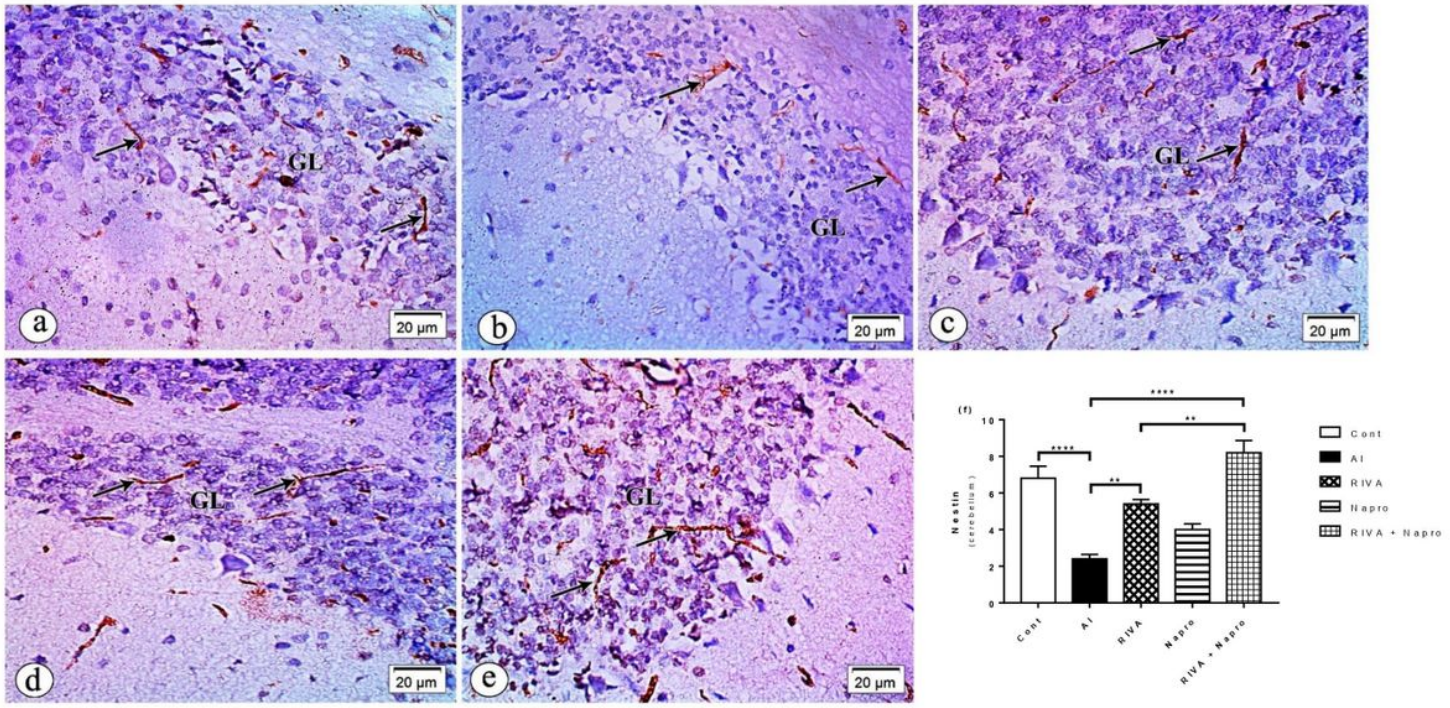

Figure 8

Photomicrographs of nestin immunostained sections of rat cerebellum. a) Cont; multiple nestin immunostained cells (↑). b) Al; few nestin immunostained cells (↑). c) RIVA; multiple nestin immunostained cells (↑). d) Napro; multiple nestin immunostained cells (↑). e) RIVA+Napro; multiple nestin immunostained cells (↑) x400. f) Statistical analysis of the number of nestin immunostained cells in cerebellar tissues. The data are expressed as mean ± SEM. **p < 0. 01, ****p < 0.0001.

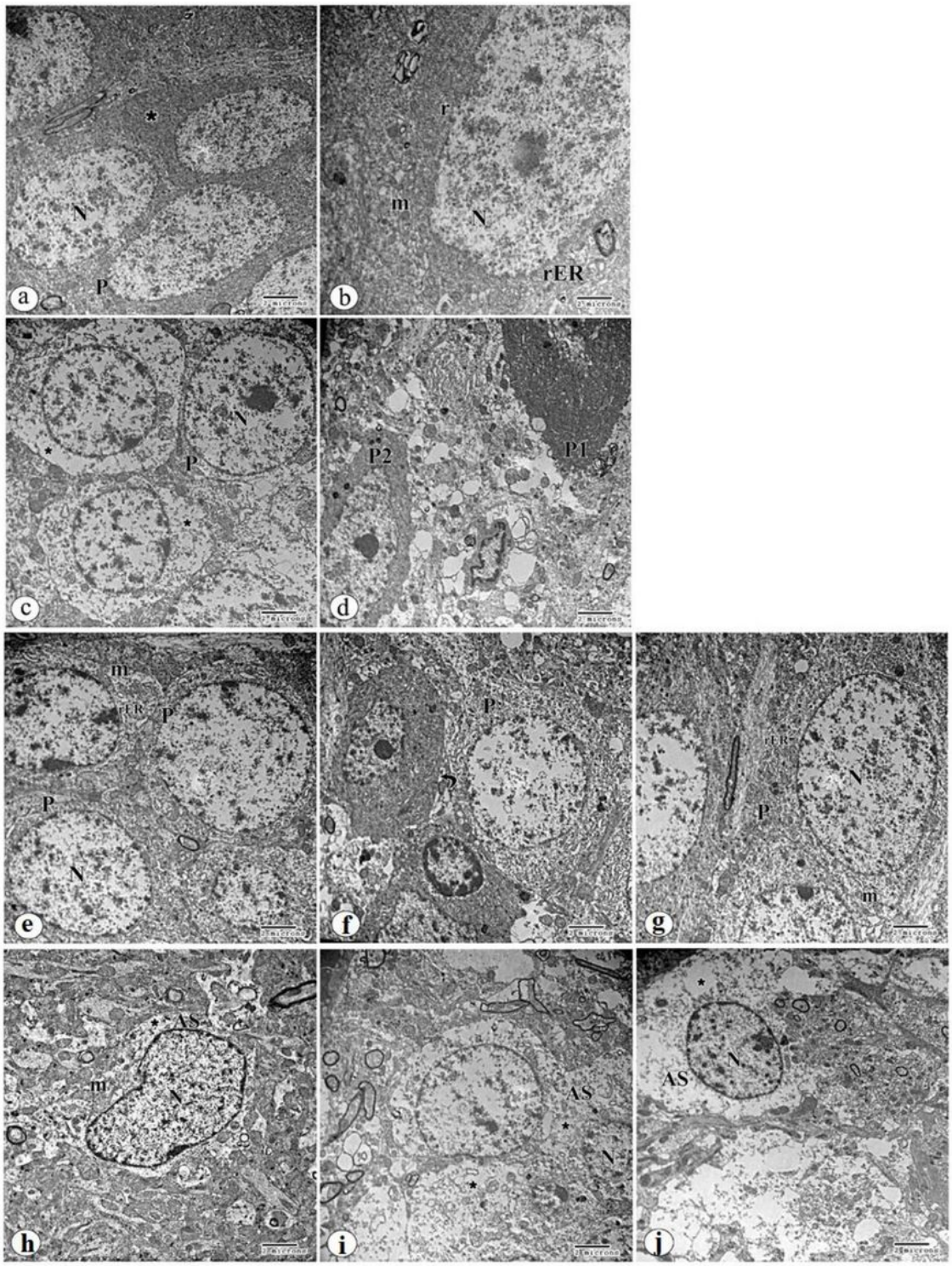

Figure 9

Transmission electron micrographs (TEM) of rat hippocampus (CA1 fields; pyramidal cells & astrocytes). a) Cont pyramidal cells; multiple medium-sized (P) with oval large nuclei (N) and abundant cytoplasm (*) x3600. b) Higher magnification shows abundant cytoplasm that contains mitochondria (m), ribosomes (r), and rER x7200. c) Al pyramidal cells; multiple (P) with euchromatic oval nuclei (N) and rarefied cytoplasm (*) x3600. d) Al pyramidal cells; electron-dense irregular cells with an ill-defined nucleus (P1)

and oval irregular nucleus (P2) x3600. e) RIVA pyramidal cells; a group of cells (P) with euchromatic oval or rounded nuclei (N), abundant electrolucent cytoplasm that contains mitochondria (m) and rER x3600. f) Napro pyramidal cells; electrolucent cells (P) and some electron-dense shrunken cells (curved arrows) x3600. g) RIVA+Napro pyramidal cells; a group of electrolucent cells (P) with oval nuclei (N), mitochondria (m), and rER x3600. h) Cont astrocytes; cells (As) have a large oval nucleus (N) and minimal cytoplasm (*) that contain mitochondria (m) x3600. i) Al astrocytes; multiple cells (AS) show electrolucent swollen cytoplasm that contains mitochondria and rounded nucleus (N) x3600. j) RIVA+Napro astrocytes; cell (AS) with swollen electrolucent cytoplasm (*) and large rounded nucleus (N) x3600.

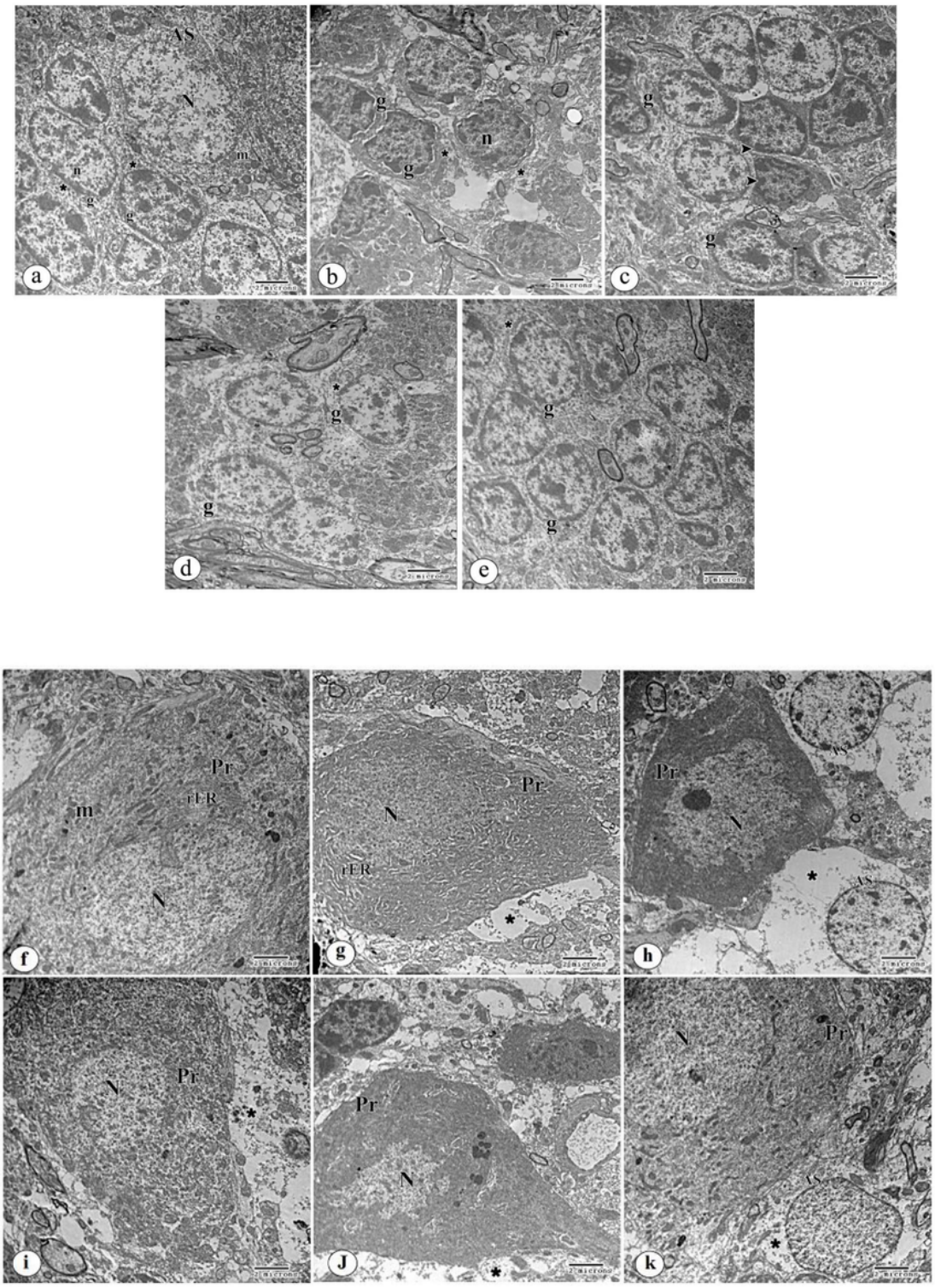

Figure 10

Transmission electron micrographs (TEM) of rat cerebellar cortex (granular & Purkinje cell layers). a) Cont granular cell layer; multiple granule cells (g), they have oval nuclei (n) and minimal cytoplasm (*). Astrocyte (AS) with a large oval nucleus can be seen (N) x3600. b) Al granular cell layer; a group of granule cells (g) with heterochromatic nuclei (n) and minimal rarified cytoplasm (*). c) RIVA granular cell layer; group of granule cells with minimal electrolucent cytoplasm (g) and some still dense and

heterochromatic nuclei (arrowheads) x3600. d) Napro granular cell layer; multiple granule cells (g) with minimal cytoplasm (*). e) RIVA+Napro granular cell layer; a group of granule cells (g) with oval nuclei and minimal cytoplasm (*) x3600. f) Cont Purkinje cell layer; Purkinje cell (Pr) perikaryon with large euchromatic nucleus (N) and voluminous cytoplasm that contains multiple mitochondria (m) and rER x3600. g) Al Purkinje cell layer; shrunken degenerated electron-dense Purkinje cell (Pr) with an ill-defined nucleus (N) and dilated rER, the surrounding neutrophils appear empty (*). h) Al Purkinje cell layer; shrunken degenerated electron-dense Purkinje cell (Pr) with a defined nucleus (N). Multiple Astrocytes (AS) with swollen cytoplasm (*) x3600. i) RIVA Purkinje cell layer; Purkinje cells (Pr) with defined nucleus (N) and voluminous cytoplasm (*) x3600. j) Napro Purkinje cell layer; degenerated electron-dense Purkinje cells (Pr) with a small irregular nucleus (N) and surrounded by degenerated neutrophils (*). k) RIVA+Napro Purkinje cell layer; part of Purkinje cell perikaryon (Pr) with large oval euchromatic nucleus (N) and electrolucent cytoplasm. Astrocyte (AS) with swollen cytoplasm (*) can be seen x3600.