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REVIEW ARTICLE

Unexpected Effects of Acetylcholine Precursors on Pilocarpine Seizure-Induced Neuronal Death

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Abstract: ***Background*:** Choline alfoscerate (α-GPC) and Cytidine 5'-diphosphocholine (CDP-Choline) are both acetylcholine precursors and are considered to act as pro-cholinergic nootropic agents. Acetylcholine precursors have also recently found frequent use in the neurology clinic. Stroke and many types of dementia have been shown to respond favorably after treatment with these agents, not only in terms of cognitive dysfunction but also behavioral and psychological symptoms. The primary mechanisms of Acetylcholine precursors are the following: 1) Acetylcholine precursors themselves are used in the biosynthesis of acetylcholine and 2) byproducts like glycerophosphate have protective functions for neuronal phospholipids. However, whether acetylcholine precursors have a similar effect in treating cognitive impairment in patients with epilepsy remains controversial.

***Methods*:** Our previous studies investigating acetylcholine precursors in seizure-experienced animals have produced variable results that were dependent on the timing of administration.

***Results*:** Early administration of CDP-choline immediately after seizure increased neuronal death, blood-brain barrier (BBB) disruption and microglial activation in the hippocampus. However, administration of α-GPC starting 3 weeks after seizure (late administration) improved cognitive function through reduced neuronal death and BBB disruption, and increased neurogenesis in the hippocampus.

***Conclusion*:** These seemingly contradictory results may be attributed to both epileptogenic features and neuroprotective functions of several acetylcholine precursors.

Keywords: Epilepsy, acetylcholine precursor, choline alfoscerate, cytidine 5'-diphosphocholine, neuron death, neurogenesis.

1. INTRODUCTION

Epilepsy is a common neurological disorder, which is characterized by recurrent unprovoked seizures [1, 2]. The syndrome can induce lasting and devastating changes across a wide spectrum of modalities, including behavioral, emotional, psychiatric and cognitive problems [3]. Among them, temporal lobe epilepsy is known to be one of the leading causes of cognitive impairment [4].

During convulsive seizure, patients may experience a variety of symptoms including partial to generalized tonic, clonic or tonic-clonic seizures, accompanied by brief loss of consciousness and even hypoxemia. Severe and sustained seizures may lead to more serious events, such as hypoxia and excitotoxic neuronal damage when not treated properly. Furthermore, even timely intervention with anti-epileptic drugs has failed to prevent delayed neuronal death, which is correlated with cognitive decline [5-7]. For this reason, numerous *in vivo* studies have tested a multitude of neuroprotective agents for the prevention of progressive neuronal damage and subsequent cognitive impairment. However, results have been inconsistent and difficult to interpret [5, 7-10]. Therefore, continuous effort is needed to develop new, more effective treatment options.

Various ways to alleviate the cognitive sequelae of recurrent epileptic fits have been investigated and a number of molecular and cellular pathways, including synthesis and secretion of neurotransmitter, have been identified as playing critical roles. Recurrent epilepsy may develop because of an imbalance in inhibitory (GABA) and excitatory neurotransmitters (glutamate). The first-line of therapies are benzodiazepines (diazepam or lorazepam), which potentiate the inhibitory neuronal responses mediated by GABA-A receptors [11]. Pilocarpine-induced seizure is initiated by cholinergic hyperactivation, but the recurrent aspect of epilepsy or propagation of seizure activity is caused by a glutamatergic

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mechanism. Neuronal death and recurrent seizure activity occurs secondary to seizure-induced glutamate release from the presynaptic terminals. Several glutamate receptor antagonists demonstrated neuroprotective effects after pilocarpine-induced seizure [12, 13]. Acetylcholine (ACh) is a neurotransmitter implicated in seizure generation [14, 15] and moreover it is involved in cognitive function. A compelling set of studies have demonstrated that electrical stimulation permanently increased hippocampal seizure responsiveness to cholinergic stimuli [16, 17], which is caused by the increased activity of the G-protein coupled muscarinic receptor activation [18]. N-Methyl-D-aspartate (NMDA) glutamate receptors are central contributors to excitotoxicity after various brain injuries, including in seizure generation, and have been also reported to associate with muscarinic ACh receptors [19, 20]. Therefore, these studies indicate that the activation of cholinergic receptors may have adverse effects on various types of seizure. One core pathway that impinges on the pathophysiology of epilepsy is that of acetylcholine biosynthesis and neurotransmission, which has only recently been investigated in the field of epilepsy research [5, 7]. Since cholinergic decline is known to be involved in the pathogenesis of Alzheimer's disease and cognitive dysfunction after trauma or cerebrovascular attack, both acetylcholinesterase inhibitors and acetylcholine precursors are being readily used in the aforementioned fields [21-25]. Therefore, the use of choline precursors in Alzheimer's disease requires special attention if the patients also have epilepsy.

Acetylcholine precursors such as choline alfoscerate (α-GPC) and cytidine 5'-diphosphocholine (CDP-Choline) are important intermediates in the synthesis of both acetylcholine and cell membrane phospholipids. The ability of acetylcholine precursors to reduce or prevent neuronal insult has been tested both in clinical and animal studies in the field of stroke and Alzheimer's disease [21, 23, 26, 27]. Clinical studies have demonstrated that cholinergic precursors improved from cognitive impairment in Alzheimer's disease and vascular dementia [28, 29]. Combination of cholinesterase inhibitors and a cholinergic precursor α-GPC increases brain acetylcholine levels and improves cognitive function in Alzheimer's patients with concomitant cerebrovascular damage [21]. Clinical trials showed that CDP-choline also improved stroke and traumatic brain injury related symptoms including motor and cognitive recovery [30-33]. However, recent studies have produced inconsistent results in seizure-experienced animals given acetylcholine precursors that varied according to the timing of administration. Treatment with CDP-choline at early time points following seizure increased neuronal death and cognitive dysfunction. However, administration of α-GPC starting 3 weeks after seizure improved cognitive function through reduced neuronal death and BBB disruption, and increased neurogenesis in the hippocampus [5-7]. These rather contradictory results may be attributed to both epileptogenic features and neuroprotective properties of acetylcholine precursors.

Currently, there are a limited number of studies that have provided evidence of the neuroprotective effects of cholinergic precursors after seizure. An interesting study has demonstrated that an acetylcholine precursor citicoline has an anticonvulsant effect on pentylentetrazole induced seizures in rats [34]. Karpova *et al*. also showed the inhibitory effects of citicoline on pentylentetrazole-induced epileptic activity in a mouse model of epilepsy [35]. In addition, cholinergic depletion showed adverse effects on epilepsy-induced cognitive decline. Cholinergic depletion in the hippocampus showed increased seizure severity and impaired spatial memory in kainate-induced seizure experienced rats [36]. Animal models of Alzheimer's disease have demonstrated increased susceptibility to seizures and these animals showed increased hippocampal damage after kainate-induced seizures [37, 38]. This increased susceptibility may be due to decreased levels of ACh in these mice [39-41]. Thus, maintenance of cholinergic activity in Alzheimer's patients is important if the patients have epilepsy. This result suggests that normal physiological cholinergic activity is needed not only for cognitive function but also for suppression of seizure susceptibility in Alzheimer's patients.

This review describes the cholinergic pathway itself, effects of acetylcholine precursors on animal epilepsy models and the potential treatment of epilepsy-induced cognitive impairment using these agents.

1.1. Mechanism of Pilocarpine-Induced Status Epilepticus (SE)

Pilocarpine acts as a muscarinic receptor agonist and is commonly used to induce seizure activity in rodents by producing a phenotype that resembles human temporal lobe epilepsy [14]. The mechanism of action of pilocarpine-induced SE depends upon activation of the muscarinic M1 receptor [42] and seizures are maintained by activation of NMDA receptors (NMDAR following M1 receptor activation [43, 44]. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation, which is activated by phospholipase C (PLC)-dependent inositol trisphosphate (IP3) release, occur independently to induce NMDAR-mediated oxidative stress. An IP3-mediated surge in intracellular Ca^{2+} and its increased influx *via* NMDARs lead to the activation of NADPH oxidase and generation of superoxide. This results in oxidative modification of cell surface NMDARs with impairment of receptor function. The aforementioned events may trigger pathological states that lead to the local activation of neuronal nitric oxide synthase (nNOS) in close association with the NMDAR, and NADPH oxidase. Thus, Ca^{2+} influx through NMDAR channels leads to the production of superoxide *via* activation of NADPH oxidase, and Ca^{2+} uptake by mitochondria in combination with NO production triggers cell death [45, 46].

1.2. How are Acetylcholine Precursors Utilized *in vivo*?

Acetylcholine precursors have a complex mechanism of action. Acetylcholine precursors such as α-GPC and CDP-choline are choline-containing phospholipids that readily cross the blood-brain barrier. When they are absorbed, they have two distinct modes of action. First, the precursors are utilized as a substrate for acetylcholine biosynthesis and can enhance cholinergic neurotransmission [47, 48]. With concurrent use of acetylcholinesterase inhibitors, acetylcholine precursors have been proved to prolong beneficial effects on cognitive and behavioral improvements in patients with both Alzheimer's disease and vascular dementia of a mild to

moderate degree [21, 49]. Other than being used directly as precursors, they may also exert substantial neuroprotective effects by maintaining and supplying the membranous structure of neurons [47, 50, 51]. Previous studies have also demonstrated increased neurogenesis in rats when acetylcholine precursors were administered 3 weeks after pilocarpine-induced seizure, implying the that acetylcholine precursors can be used not only as a neuroprotective measure but also to facilitate neurogenesis after ischemic or epileptic insult [5, 52]. In a recent human clinical trial, α-GPC showed neuroprotective effects by preventing brain atrophy [21].

1.3. Effects of Acetylcholine Precursors in Animal Epilepsy Models

Administration of acetylcholine precursors in degenerative, vascular or traumatic brain injury models is based on the hypothesis that acetylcholine levels are pathologically low in these settings. They act as a donor for acetylcholine itself in pre-synaptic neurons to promote normal cholinergic neurotransmission and ultimately maintain cognitive and behavioral function [21]. However, as high-levels of acetylcholine are known to lower the threshold for seizure, acetylcholine precursors may worsen the prognosis of epileptic

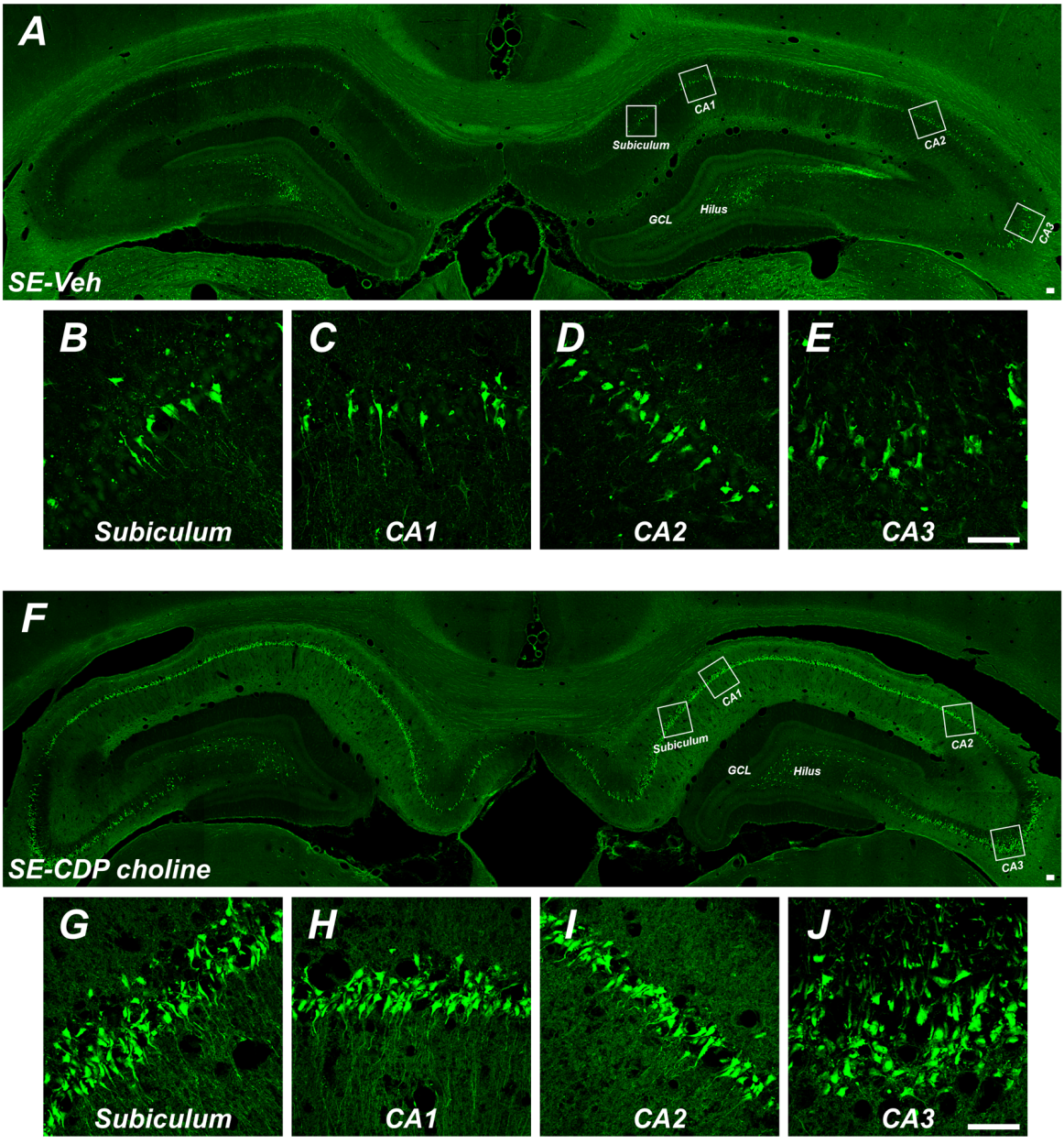

Fig. (1). Early administration of CDP-choline aggravates pilocarpine-induced neuronal death in the hippocampus. Fluorescent photomicrographs indicate degenerating neurons stained by Fluoro-Jade B (FJB, green) in the various hippocampal areas, such as subiculum (**B**, **G**), CA1 (**C**, **H**), CA2 (**D**, **I**) and CA3 (**E**, **J**) at 1 week after seizure with (**A**-**E**) or without CDP-choline (**F**-**J**). CDP-choline administered into the intraperitoneal space as a choline donor once per day for 1 week after seizure and provided adverse effects on hippocampal neuronal death, compared to vehicle-treated group. Scale bar = 50μm. (*The color version of the figure is available in the electronic copy of the article*).

patients or animals when given acutely [53]. Furthermore, previous studies have reported that acetylcholine levels are elevated following status epilepticus [54].

However, even upon achieving control of chronic seizures, delayed neuronal damage and subsequent cognitive decline often occur. During this period when the outward appearance of seizure is controlled, administration of acetylcholine precursors may promote neurogenesis and prevent further neuronal damage, which can result in an overall improvement in cognition and behavioral outcomes [5].

1.4. Early Administration of Acetylcholine Precursors Adversely Effects Seizure-Induced Neuronal Death

CDP-choline was used to clarify the effects of early administration of acetylcholine precursors on seizure-induced neuronal death. We used an animal model of pilocarpine-induced epilepsy. Seizures were induced by intraperitoneal injection of pilocarpine (25mg/kg) in adult male rats. CDP-choline was injected into the intraperitoneal area two hours after seizure induction and maintained on a once per day regimen for one week. Neuronal death and microglial activation were evaluated at 1 week after seizure. CDP-choline treatment resulted in aggravated neuronal death, which was evidenced by pronounced Fluoro-Jade B staining (Fig. **1**). Furthermore, the CDP-choline treated group showed signs of greater microglial activation and BBB disruption in the hippocampus, compared to the vehicle-treated groups [7].

1.5. Elevated Levels of Acetylcholine and Glutamate may be Associated with the Adverse Effects of Acetylcholine Precursors on Seizure-Induced Neuronal Death

Previous studies using microdialysis to monitor the levels of extracellular glutamate and acetylcholine before, during

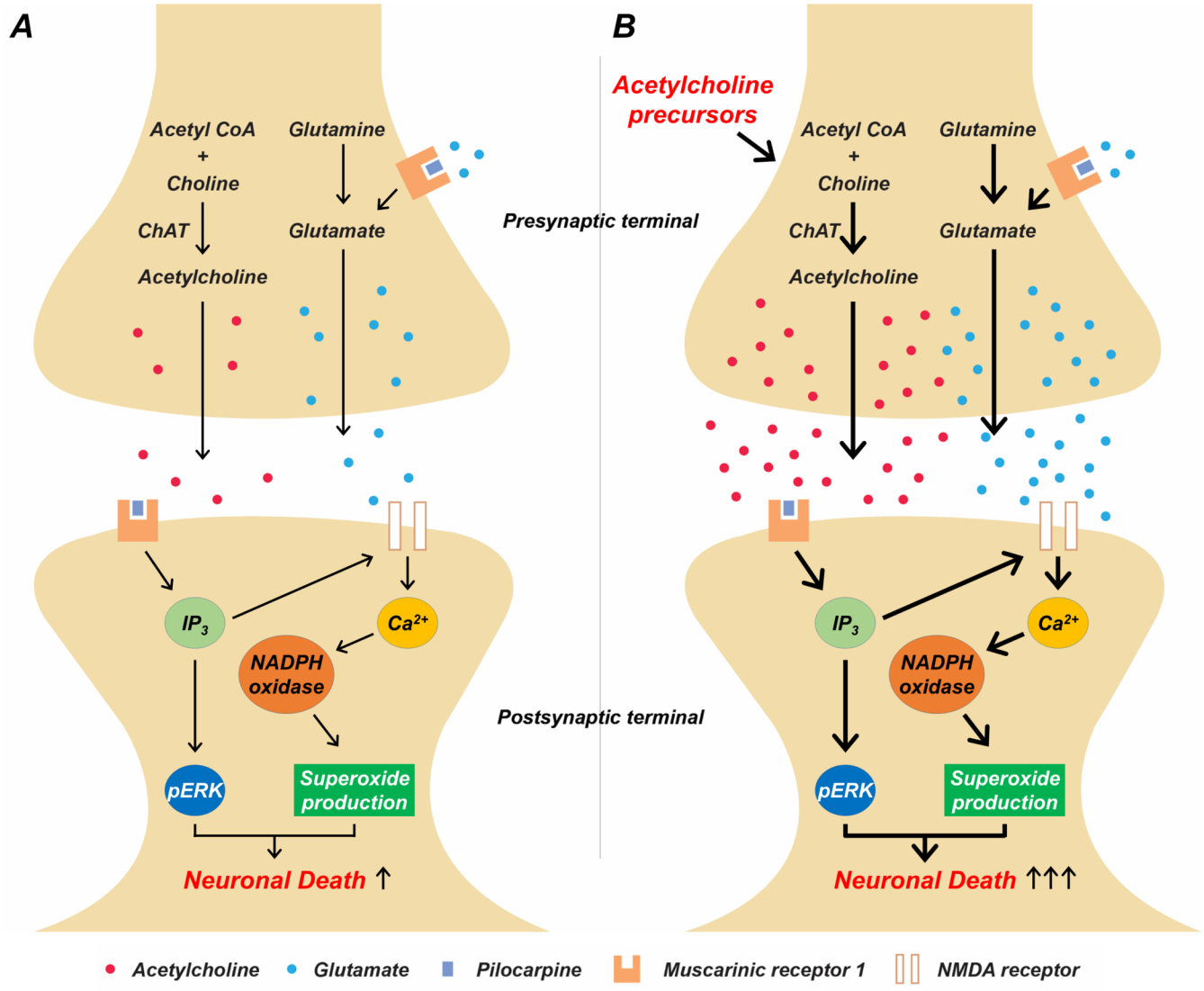

Fig. (2). Possible association of acetylcholine level with pilocarpine-induced neuronal death. This schematic drawing indicates several chain reactions that may occur after acetylcholine precursor treatment in pilocarpine-induced SE. (**A**) These are mechanism of pilocarpine-induced neuronal death. (**B**) Increased acetylcholine levels due to administration of acetylcholine precursors aggravates pilocarpine-induced neuronal death.

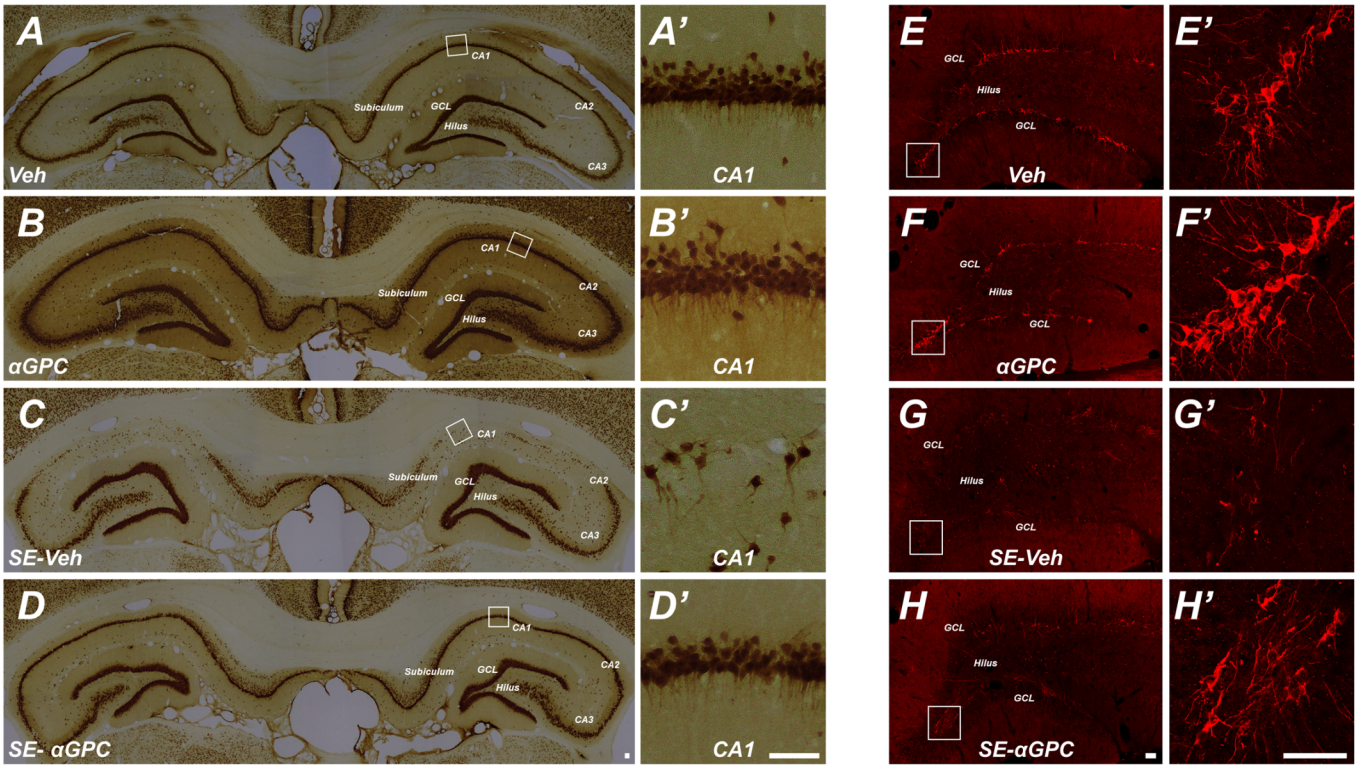

Fig. (3). Late administration of α-GPC reduces neuronal death and increases neurogenesis after seizure. (**A-D**) Neuronal nuclei (NeuN) immunohistochemistry in the hippocampus. Inset areas depicting details from CA1 (') are presented in higher magnification with corresponding group labels. Control groups that received vehicle (**A**) or α-GPC (**B**) injection represent the expected NeuN distribution with typically dense granular and pyramidal layers. Note the neuronal loss in the SE group (**C**) compared to control groups (**A**), where relatively few neurons can be observed in the CA1 regions (**C'**). α-GPC treatment remarkably ameliorated neuronal loss in the hippocampus after seizure. Note that relative to the SE group (**C**), more cells are present in the α-GPC treated SE group in CA1 (**D'**). (**E-H**) Doublecortin (DCX), a neuroblast marker, immunofluorescence staining in dentate gyrus (DG) of the hippocampus. Higher magnification of all treatment groups are shown in subgranular zone (SGZ) of DG (**E'-H'**). At 6 weeks after seizure, the number of DCX-positive cells was significantly decreased in the SE group (**G**) compared to the control group (**E**). α-GPC treatment remarkably increased the number of DCX-positive cells in the DG after seizure. Note that relative to the SE group (**G**), more neuroblasts are present in the α-GPC treated SE group in the SGZ (**H'**). Scale bar = 50μm.

and after pilocarpine-induced seizure have revealed the following: 1) The onset of status epilepticus after pilocarpine induction is accompanied by a massive increase in extracellular acetylcholine and 2) treatment of seizure activity with either ketamine or diazepam reduces the levels of acetylcholine [55]. 3) Additionally, systemic injection of pilocarpine is known to act on pre- and post-synaptic muscarinic receptors and increases glutamate levels prior to the development of epileptic seizure, which was also accompanied by increased acetylcholine levels [56]. Taken together, administration of acetylcholine precursors at early time points (~2 hours) in the setting of pilocarpine-induced status epilepticus may further elevate acetylcholine levels, which were already observed to increase several-fold due to pilocarpine administration alone. This synergistic elevation of acetylcholine due to early administration of acetylcholine precursors may further increase the levels of glutamate, which can further aggravate excitotoxic neuronal death (Fig. **2**). Additional microdialysis studies should be conducted to provide additional evidence for this hypothesis.

1.6. Late Administration of Acetylcholine Precursors Provides Protection Against Seizure-Induced Neuronal Death

Our previous study also tested the potential therapeutic effects of α-GPC on pilocarpine seizure-induced neuronal death when administered at later time points. Seizure was induced by injection of pilocarpine (25mg/kg) in male rats. α-GPC was injected once daily from 3 weeks after the seizure onset for 3 additional weeks. The rats were subjected to a water maze test and then sacrificed. In order to verify the beneficial effects for preventing neuronal death and BBB disruption, we performed NeuN and IgG staining at 6 weeks after seizure. A clear increase in the number of NeuN-positive neurons in the α-GPC late treatment group (compared to the vehicle group) indicated that the number of live neurons in the hippocampal area was enhanced with treatment (Fig. **3A-D**).

Moreover, the late α-GPC treatment group showed a significant decrease in IgG extravasation; a strong indicator

that BBB disruption was reduced. Additionally, administration of α-GPC promoted improved performance in a standard water maze test protocol compared to the vehicle-treated group. These results suggest that late treatment of α-GPC improved cognitive function through reduced neuronal death and BBB disruption [5].

1.7. Late Administration of Acetylcholine Precursors Increases Hippocampal Neurogenesis After Seizure

In the aforementioned studies, we performed immunohistochemical staining with an anti-doublecortin antibody (DCX) to identify the number of active neuroblasts. Late administration of α-GPC showed an increase in number and dendritic intensity of DCX-positive cells in the subgranular zone of the dentate gyrus, when compared to the vehicle-treated group (Fig. **3E-H**). This result implies that acetylcholine precursors may increase the production of immature neurons when administered at time points later than 3 weeks after seizure [5].

CONCLUSION

Acetylcholine precursors may play several crucial roles in neuronal death, microglial activation, BBB disruption and consequent cognitive impairment after epileptic seizure as a function of their time of administration relative to insult. Further investigation into pathophysiological mechanisms of acetylcholine precursors in epilepsy models and methods for alleviating cognitive impairment in clinical settings are highly warranted.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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