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The novel GLP-1/GIP dual receptor agonist DA3-CH is neuroprotective in the pilocarpine-induced epileptogenesis rat model

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ABSTRACT

Aims: Glia-mediated neuro-inflammation and oxidative stress-induced neuronal apoptosis can contribute to epileptogenesis. We have demonstrated previously that mimetics of glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and dual-GLP-1/GIP receptor agonists protect the brain from inflammation, oxidative stress, apoptosis and neuronal loss in animal models of central nervous system diseases. *Methods:* This study investigated for the first time whether the novel dual GLP-1/GIP receptor agonist DA3-CH has neuroprotective effects in the pilocarpine-induced status epilepticus (SE) rat model and the studies the underlying mechanisms. DA3-CH was administered once daily at 10 nmol/kg ip. following SE induction. The effect of DA3-CH was evaluated by immunohistochemistry and western blot at 12 h, 1 d, 3 d, 7 d after kindling. *Results:* Our findings show that DA3-CH reduced the chronic inflammation response (astrogliosis and microgliosis), and the associated release of the pro-inflammatory cytokines interleukin-1β (IL-β) and tumor necrosis factor-α (TNF-α) in the hippocampal CA1 area. Furthermore, DA3-CH reduced the expression of the mitochondrial pro-apoptotic protein Bax, while increasing the expression of the anti-apoptotic protein Bcl-2. Neuronal numbers in the CA1 area were much reduced by pilocarpine treatment, and DA3-CH protected neurons from neurotoxicity.
Conclusion: These results demonstrated that DA3-CH could mitigate pilocarpine-induced neuro-inflammation, mitochondrial apoptosis and neuronal loss. The findings encourage the development of dual agonists as novel therapeutic interventions for epilepsy.

1. Introduction

Epilepsy is one of the most common neurological disorders worldwide, which affects up to 1% of the world population (Mendez-Armenta et al., 2014). The median prevalence of lifetime epilepsy is 5.8 per 1000 for developed countries and 10.3 per 1000 for developing countries (Ngugi et al., 2010). With diverse etiology, epilepsy is characterized by spontaneous recurrent seizures (SRS) due to hyperexcitability and hypersynchrony of neurons in the brain. As the most grievous form of an epileptic seizure, status epilepticus (SE) is a major neurological and medical emergency that is associated with significant morbidity and mortality (AM and SR, 1980; Trinka et al., 2015). However, epidemiological studies shown that antiepileptic drugs (AEDs) are ineffective or give rise to unacceptable adverse reactions in approximately one third of epilepsy patients (Fisher et al., 2005; Si et al., 2016). At present, most studies on the effects of AEDs focus on neurons, ion channels and transporters, as well as excitatory and inhibitory neurotransmission, which seems to only influence the acute process of ictogenesis (i.e., induction of an acute seizure), but not modify the underlying SRS (Devinsky et al., 2013). In addition, they do not address the neurodegenerative processes that are initiated by SE. Clearly, there is an impending need to explore novel therapeutic strategies for prevent the emergence of SRS.

Epileptogenesis is defined as the latent period before which SRS occur (Biagini et al., 2013), which is a process whereby a brain becomes progressively epileptic due to diverse initial destabilizing events such as brain injury, stroke, infection, or prolonged seizures (Reddy, 2013). The traditional molecular mechanisms of AEDs may be more connected with ictogenesis than epileptogenesis, the latter reflecting a variable process leading to a continual state of SRS (Loscher and Brandt, 2010). Therefore, we explore experimental interventions designed to arrest or modify the epileptogenic process. More specifically, the alterations

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Table 1
ANOVA results of the different analyses of biomarkers.

Result	F-value	DF	p value
Glucose	0.14	16	ns.
GFAP	43.34	8	0.0001
IBA-1	32.22	8	0.0001
IL-1ß	66.4	8	0.0001
TNF-α	286.5	8	0.0001
BAX	34.08	8	0.0001
Bcl-2	151.2	8	0.0001
Histology results:			
NeuN	52.7	8	0.0001
GFAP	195.9	8	0.0001
IBA-1	278.3	8	0.0001

during the epileptogenetic process in animal models include glial cell activation, neuroinflammation, neuronal injury and cell death, axonal and dendritic plasticity, presynaptic and postsynaptic modifications, neurogenesis, vascular damage and angiogenesis, disruption of extracellular matrix integrity, as well as structural (i.e., subunit) and functional changes in ion channels properties (Kobow et al., 2012). In general, one or more therapeutic interventions based on such changes could potentially have protective effects on epileptogenesis (Table 1).

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones that could promote glucose-dependent insulin secretion and inhibit glucagon secretion (Baggio and Drucker, 2007; Campbell and Drucker, 2013; Finan et al., 2013). Mimetics of GLP-1 and GIP can cross the blood-brain barrier (BBB) and respectively activate GLP-1R and GIPR that widely expressed in the brain to promote of nerve cell growth, proliferation, differentiation and repair as well as inhibit glial cell activation, neuroinflammation, oxidative stress and apoptosis (D-i and Park, 2015; Holscher, 2014a; Darsalia et al., 2012; Faivre and Hölscher, 2013). GLP-1R and GIPR single agonists have shown neuroprotective effects in animal models or patients of central nervous system (CNS) disease, such as Alzheimer's disease (AD), Parkinson's disease (PD) and stroke (Faivre and Hölscher, 2013; Duffy and Hölscher, 2013; Faivre and Holscher, 2013; McClean et al., 2011; Ji et al., 2016; Bertilsson et al., 2008; Harkavyi et al., 2008; Li et al., 2009; Liu et al., 2015; Zhang et al., 2015; Li et al., 2016; Holscher, 2014b; Michael et al., 2016; Hölscher, 2016; Aviles-Olmos et al., 2013, 2014; Athauda et al., 2017; Hölscher, 2018). We have tested the GLP-1 analogue liraglutide in the SE rat model previously and it showed good neuroprotective effects. Chronic inflammation and apoptosis in the brain was much reduced (Wang et al., 2018). We furthermore demonstrated that novel dual-GLP-1/GIP receptor agonists have greater neuroprotective effects compared with single GLP-1 analogues. In a first study, we tested a novel dual GLP-1/GIP receptor agonist that was more protective against functional decline than the GLP-1 analogue Val(8)-GLP-1-(glu-PAL) in the transient focal cerebral ischemia rat model by reducing the chronic inflammatory response and cellular apoptosis. (Han et al., 2016). We furthermore tested a dual GLP-1/GIP receptor agonist (DA-JC4) that alleviates learning and memory deficits by decreasing phosphorylated tau protein levels, chronic inflammation response and apoptosis as well as re-sensitizing insulin signaling in the Alzheimer icv. streptozotocin (STZ) rat model (Shi et al., 2017). In addition, we tested the GLP-1/GIP dual agonist DA3－CH in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, which showed enhanced neuroprotective properties over the single GLP-1 receptor agonist liraglutide in Inhibiting the chronic inflammation response, increasing the expression levels of the neuroprotective growth factor Glial Derived Neurotrophic Factor (GDNF), protecting mitochondria, rescuing impaired dopamine synthesis, and improving dyskinesia (Hölscher, 2018; Lovshin and Drucker, 2009; Yuan et al., 2017). DA3－CH had clear neuroprotective effects in a mouse model of Alzheimer's disease. Memory formation was improved, the amyloid plaque load reduced, and autophagy normalised (Panagaki et al., 2018). Other dual receptor agonists also had neuroprotective effects in animal models of Parkinson's or Alzheimer's disease (Feng et al., 2018; Cao et al., 2018). From these studies, we learned that GLP-1/GIP dual agonists can inhibit neuroinflammation mediated by glial cells and neuronal death induced by oxidative stress that contribute to epileptogenesis (Mendez-Armenta et al., 2014; Devinsky et al., 2013; Wang et al., 2015).

As these drugs have shown clear neuroprotective effects in Alzheimer and Parkinson's disease animal models, we aimed to investigate whether the GLP-1/GIP dual agonist DA3－CH demonstrates protective effects in the pilocarpine-induced SE rat model, and can influence the underlying pathological mechanisms. We monitored the timeline of microglial and astrocytic activation that plays a critical role in the initiation and maintenance of neuroinflammation, and analyzed key markers of mitochondrial apoptosis, and studied the neuronal loss in the hippocampal CA1 area following pilocarpine treatment.

2. Materials and methods

2.1. Animals

Healthy adult male Sprague-Dawley (SD) rats weighing 250–300 g were supplied by the Beijing Vital River Laboratory Animal Technology (Beijing, China). All the rats were housed under controlled environmental conditions (22 ± 3 °C, 50–55% humidity and a 12:12-h light/dark cycle) with fast 8 h before measuring blood glucose. All experimental protocols were approved by the Ethics Committee of Shanxi Medical University and performed in accordance to National Institute of Health (NIH) guideline (NIH Publication, No. 80-23, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

2.2. DA3-CH

The dual agonist DA3-CH was synthetized by Chinapeptides Ltd (Shanghai, China). The purity of the peptide was confirmed by reversed-phase HPLC and characterized using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry.

DA3-CH was freshly dissolved in 0.9% normal saline to a concentration of 1 mg/40 mL and administered intraperitoneally once daily just before the animals' meals at a dosage of 10 nmol/kg rat body weight, starting immediately after termination of SE.

Peptide sequence of the GLP-1/GIP dual agonist DA3-CH (Finan et al., 2013; Yuan et al., 2017):

YXEGTFTSDYSIYLDKQAAXEFVNWLLAGGPSSGAPPPSK-NH2

X = aminoisobutyric acid

2.3. Induction of status epilepticus (SE)

The experimental rats were injected intraperitoneally with lithium chloride (127 mg/kg, Sigma-Aldrich, USA) followed by pilocarpine (30 mg/kg, MedChem Express, USA) 20 h later. The rats were pretreated with atropine sulfate (1 mg/kg, ip., Tianjin Jinyao, China) 30 min before pilocarpine injection to reduce the peripheral cholinomimetic effects of pilocarpine. Behavioral changes were recorded and graded in each rat according to Racine's standard stages (RR, 1972). Rats in stages 4 or 5 for more than 30 min without regaining normal behavior between convulsive seizures were considered successfully kindled. To terminate convulsions, the animals were injected with diazepam (10 mg/kg, ip., Tianjin Jinyao, China) at 1 h post-seizure. Rats in the control group were intraperitoneally injected with the same volume of normal saline (NS) as the volume of pilocarpine injections.

2.4. Experimental protocol

A total of 54 SD rats were randomly divided into three groups: (1)

normal saline treated (control) group (NS, rats without SE, n = 6), (2) DA3-CH treated group (SE + DA3-CH, n = 24), which received DA3-CH immediately after termination of SE, followed by once daily DA3-CH treatment (10 nmol/kg, ip.) for the subsequent period, (3) status epilepticus group (SE, n = 24), which received the same volume of normal saline (NS) instead of DA3-CH once daily. DA3-CH-treated group and status epilepticus group respectively composes of 4 subgroup (n = 6 per subgroup), with each subgroup corresponding to a time period after the onset of status epilepticus: 12 h, 1 d, 3 d, 7 d. The rats were sacrificed to take out the brain tissue at 12 h, 1 d, 3 d, 7 d after kindling.

2.5. Blood glucose measurements

We collected blood samples from the tail vein served as the fasting blood glucose measurement. Blood glucose levels were measured by the Sannuo blood glucose meter (Sinocare Inc. China) before treatment and before sacrifice at different time points (12 h, 1 d, 3 d, 7 d after the onset of SE). Levels are reported as mmol/l.

2.6. Immunohistochemistry (IHC)

The rats (n = 3 per subgroup) were anesthetized with 5% chloral hydrate (5 ml/kg, ip.), then transcardially and successively perfused with cold 0.9% Nacl and chilled 4% paraformaldehyde (PFA). The brains were isolated and fixed in 4% PFA at 4 °C for 12 h. After dehydrated with gradiented alcohol and transparentized with xylene by automatic biological-tissue hydroextractor (Leica, Germany), the brains were embedded in paraffin. Serial coronal 5 μm sections were cut with a microtome (Leica, Germany). The sections were incubated at 37 °C for 2 h with the following rabbit anti-rat primary antibodies: GFAP (1:2000, Abcam, UK), Iba-1 (1:8000, Abcam, UK) and NeuN (1:3000, Abcam, UK), and then with secondary antibody, goat anti-rabbit IgG labeled with horseradish peroxidase (HRP) (ZSGB-BIO, China), for 30 min at 37 °C. The staining was visualized with DAB kit (ZSGB-BIO, China). All sections were also stained with hematoxylin. Images of hippocampal CA1 area were observed with a microscope (Leica DM1000, Germany) and captured with a digital camera. According to unbiased stereological rules, three sections were taken from each brain tissue, and six non-overlapping fields were randomly selected from each section for automated counting of positive cells with Image-Pro Plus 6.0 (Media Cybernetics, USA). The mean obtained from images of each rat was used for statistical analysis. All analyses were blinded.

2.7. Western blots (WB)

The rats (n = 3 per subgroup) were anesthetized with 5% chloral hydrate (5 ml/kg, ip.), and then transcardially perfused with cold 0.9% NaCl to flush away the blood. The hippocampi were isolated from brains and homogenised with RIPA lysis buffer (Beyotime, China) added phenyl-methylsulfonyl fluoride (PMSF) (Beyotime, China) on ice. After homogenates were centrifuged (12,000 rpm (4000 *g*) ×10 min, 4 °C, Eppendorf 5810R, Germany), the supernatants were collected and rapidly frozen at −80 °C for analyses. The protein concentrations of the supernatants were detected with a BCA Protein Assay Kit (Boster, China). The protein samples were added with 5X loading buffer (Boster, China) to the same concentration and denatured with boiling water for 8 min. A total of 20 μg of protein was loaded in each lane and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDA-PAGE) (Boster, China) at 80 V for 2 h. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 60 V for 100 min. The PVDF membranes were incubated with 5% skim milk at 37 °C for 1 h to block nonspecific binding. Next, the PVDF membranes were incubated at 4 °C overnight with the following rabbit anti-rat primary antibodies: β-actin (1:5000, Bioworld, USA), GFAP (1:10000, Abcam, UK), Iba-1 (1:1000, Abcam, UK), IL-1β (1:1000, Bioworld, USA), TNF-α (1:1000, Abcam, UK), Bax (1:1000, Bioworld, USA), and Bcl-2 (1:1000, Bioworld, USA). After washed with Tris Buffered Saline Tween (TBST) (10 min × 3), PVDF membranes were incubated with secondary antibody, goat anti-rabbit IgG conjugated to peroxidase (1:3000, Boster, China), at room temperature for 2 h and then washed with TBST (10 min × 3). The protein bands were detected by Enhanced Chemilluminescence (ECL) substrate (Boster, China) and analysed by the Image Lab 3.0 System.

2.8. Statistical analysis

Data were analyzed by one-way ANOVA with Tukey multiple comparison post-hoc tests. Data were analysed for normal distribution of the data was beforehand, and a log transformation was performed if the data were not normally distributed. The program Prism 5 (GraphPad, USA) was used. Data are shown as means ± SEM.

3. Results

3.1. Fasting blood glucose levels

The dual agonist DA3-CH had no effects on blood glucose levels in normoglycemic animals. A one-way ANOVA did not find a difference between groups when analyzing blood plasma glucose levels on day 1

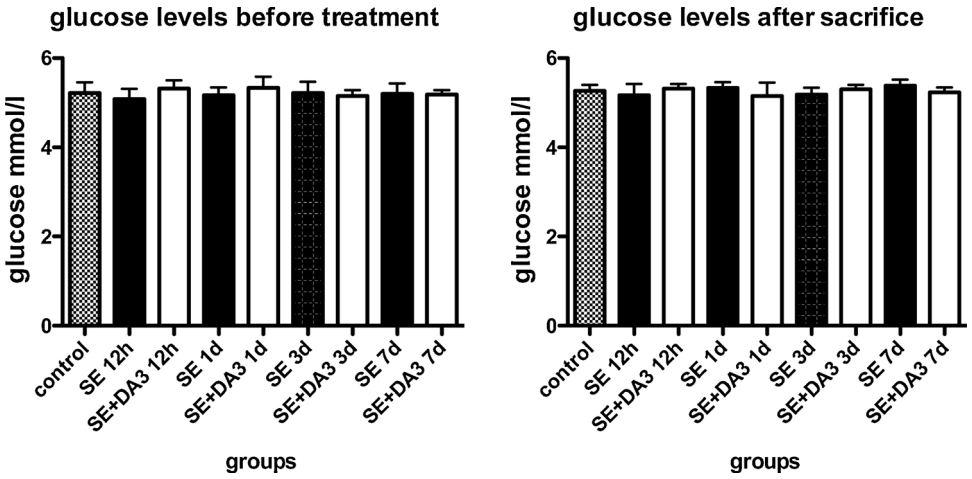
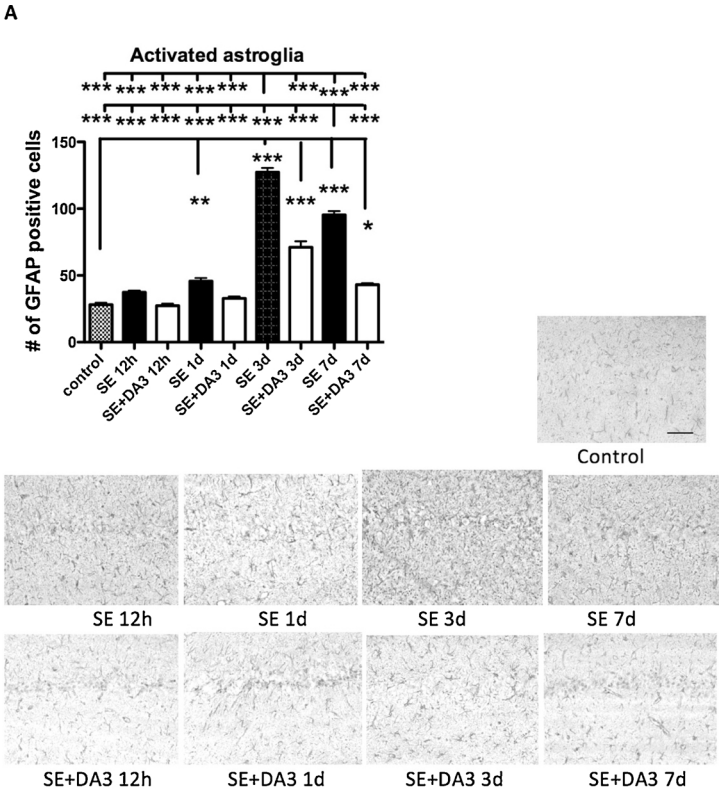


Fig. 1. Measurements of blood glucose levels. A one-way ANOVA did not find a difference between groups as measured over the duration of the experiment. N = 6.



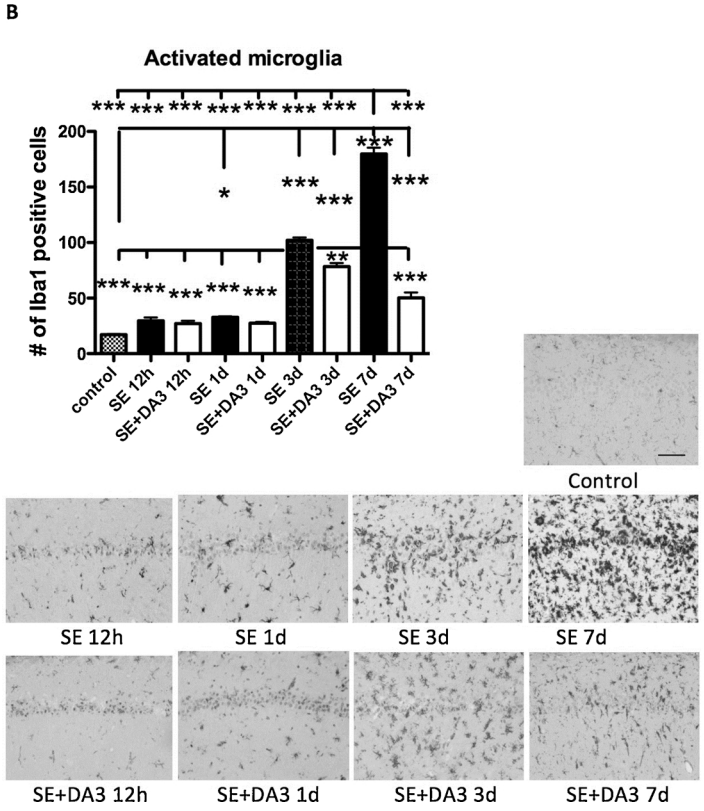


Fig. 2. The chronic inflammation response is reduced by DA3-CH, histological analysis. 2A: Histological analysis of activated astroglia (GFAP positive cells). A one-way ANOVA found a difference between all groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE at day 3 vs. all other groups, and SE at day 7 vs. all other groups. For further results, please refer to the results section. Sample micrographs are shown. 2B: Histological analysis of activated microglia (IBA-1 positive cells). A one-way ANOVA found a difference between groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE at day 3 vs. all other groups, and SE at day 7 vs. all other groups. For further results, please refer to the results section. $*=p < 0.05$; $**=p < 0.01$; $***=p < 0.001$. Sample micrographs are shown. Scale bar in control micrograph $=40\,\mu m$.

before treatment started and on day 7 when the experiment was ended (Fig. 1).

3.2. Activated microglia and astroglia (GFAP and Iba-1 positive cells)

Glial fibrillary acidic protein (GFAP) is a biomarker of astrocytic activation and of chronic inflammation in the brain. A one-way ANOVA found a difference between groups when analyzing the numbers of GFAP positive cells in area CA1 ($p < 0.0001$). Tukey post-hoc tests found significant differences between groups (see Fig. 2A). Pilocarpine greatly enhanced the number of activated astroglia compared to control animals by 4.5-fold change at 3d after SE ($p < 0.001$). Treatment with DA3−CH greatly reduced the number by 44% change ($p < 0.001$).

Ionized calcium-binding adaptor molecule-1 (Iba-1) is the biomarker for microglial activation and chronic inflammation. A one-way ANOVA found a difference between groups when analyzing the numbers of IBA-1 positive cells in area CA1 ($p < 0.0001$). Tukey post-hoc tests found significant differences between groups (see Fig. 2B). Pilocarpine greatly enhanced the number of activated microglia compared to control animals by 10.6-fold change at 7d after SE ($p < 0.001$). Treatment with DA3−CH greatly reduced the number by 72% change ($p < 0.001$). The drug treatment reduced the chronic inflammation in the brain.

GFAP:	p-value:	IL-1 IBA-1:	p-value:
control vs SE 1d	**	control vs SE 1d	*
control vs SE 3d	***	control vs SE 3d	***
control vs SE + DA3 3d	***	control vs SE + DA3 3d	***
control vs SE 7d	***	control vs SE 7d	***
control vs SE + DA3 7d	*	control vs SE + DA3 7d	***
SE 12 h vs SE 3d	***	SE 12 h vs SE 3d	***
SE 12 h vs SE + DA3 3d	***	SE 12 h vs SE + DA3 3d	***
SE 12 h vs SE 7d	***	SE 12 h vs SE 7d	***
SE + DA3 12 h vs SE 1d	**	SE 12 h vs SE + DA3 7d	**
SE + DA3 12 h vs SE 3d	***	SE + DA3 12 h vs SE 3d	***
SE + DA3 12 h vs SE + DA3 3d	***	SE + DA3 12 h vs SE + DA3 3d	***
SE + DA3 12 h vs SE 7d	***	SE + DA3 12 h vs SE 7d	***
SE + DA3 12 h vs SE + DA3 7d	**	SE 1d vs SE 3d	***
SE 1d vs SE + DA3 1d	*	SE 1d vs SE + DA3 3d	***
SE 1d vs SE 3d	***	SE 1d vs SE 7d	***
SE 1d vs SE + DA3 3d	***	SE + DA3 1d vs SE 3d	***
SE 1d vs SE 7d	***	SE + DA3 1d vs SE + DA3 3d	***
SE 1d vs SE + DA3 7d	*	SE + DA3 1d vs SE 7d	***
SE + DA3 1d vs SE 3d	***	SE + DA3 1d vs SE + DA3 7d	***
SE + DA3 1d vs SE + DA3 3d	***	SE 3d vs SE + DA3 3d	***
SE + DA3 1d vs SE 7d	***	SE 3d vs SE + DA3 7d	***
SE 3d vs SE + DA3 3d	***	SE + DA3 3d vs SE 7d	***
SE 3d vs SE + DA3 7d	***	SE + DA3 3d vs SE + DA3 7d	***
SE + DA3 3d vs SE 7d	***	SE 7d vs SE + DA3 7d	***
SE + DA3 3d vs SE + DA3 7d	***		

*=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.01$

3.3. GFAP, Iba-1 expression levels in the brain

To confirm the histology results using the western blot technique, total levels of GFAP and Iba-1 were analyzed. A one-way ANOVA found a difference between groups when analyzing GFAP ($p < 0.0001$) and Iba-1 levels ($p < 0.0001$). Tukey post-hoc tests found significant differences between groups (see Fig. 3A, 3B). The results show that DA3-CH inhibits the SE-induced glia activation.

GFAP groups:	p-value:	IL-1ßIBA-1 groups:	p-value:
control vs SE 1d	**	control vs SE 1d	**
control vs SE 3d	***	control vs SE 3d	***
control vs SE + DA3 3d	***	control vs SE 7d	***
control vs SE 7d	***	SE 12 h vs SE 3d	**
SE 12 h vs SE 3d	***	SE 12 h vs SE 7d	***
SE 12 h vs SE + DA3 3d	*	SE + DA3 12 h vs SE 1d	**
SE 12 h vs SE 7d	***	SE + DA3 12 h vs SE 3d	***
SE + DA3 12 h vs SE 1d	**	SE + DA3 12 h vs SE 7d	***
SE + DA3 12 h vs SE 3d	***	SE 1d vs SE + DA3 1d	*
SE + DA3 12 h vs SE + DA3 3d	***	SE 1d vs SE 3d	*
SE + DA3 12 h vs SE 7d	***	SE 1d vs SE + DA3 1d	*
SE 1d vs SE + DA3 1d	*	SE 1d vs SE 7d	***
SE 1d vs SE 3d	***	SE + DA3 1d vs SE 3d	***
SE 1d vs SE 7d	***	SE + DA3 1d vs SE 7d	***
SE 1d vs SE + DA3 7d	*	SE 3d vs SE + DA3 1d	***
SE + DA3 1d vs SE 3d	***	SE 3d vs SE + DA3 7d	***
SE + DA3 1d vs SE + DA3 3d	***	SE + DA3 1d vs SE 7d	***
SE + DA3 1d vs SE 7d	***	SE 7d vs SE + DA3 7d	***
SE 3d vs SE + DA3 3d	***		
SE 3d vs SE + DA3 7d	***		
SE + DA3 3d vs SE 7d	*		
SE + DA3 3d vs SE + DA3 7d	***		

*=$p < 0.05$; **=$p < 0.01$; ***=$p < 0.01$

3.4. IL-1β and TNF-α pro-inflammatory cytokine levels in the brain

Activated microglia expressed a range of pro-inflammatory cytokines, including IL-1β and TNF-α. A one-way ANOVA found a difference between groups when analyzing IL-1β ($p < 0.0001$) and TNF-α levels ($p < 0.0001$). Tukey post-hoc tests found significant differences between groups (see Fig. 3C, D). Compared to control animals, the levels of pro-inflammatory cytokines were much increased by a 5.4-fold change (IL-1β) and 4.4-fold change(TNF-α) at 7d after SE as analysed by western blot($p < 0.001$). DA3−CH reduced levels significantly by74% change (IL-1β) and 63% change (TNF-α) ($p < 0.001$). This demonstrates that the inflammation response was much reduced by the drug.

IL-1ß groups:	p-value:	IL-1ß TNF-α groups:	p-value:
control vs SE 12 h	**	control vs SE 1d	**
control vs SE 1d	***	control vs SE 3d	***
control vs SE + DA3 1d	**	control vs SE + DA3 3d	***
control vs SE 3d	***	control vs SE 7d	***
control vs SE 7d	***	control vs SE + DA3 7d	***
SE 12 h vs SE 3d	***	SE 12 h vs SE 1d	*
SE 12 h vs SE 7d	***	SE 12 h vs SE 3d	***
SE + DA3 12 h vs SE 1d	***	SE 12 h vs SE + DA3 3d	***
SE + DA3 12 h vs SE 3d	***	SE 12 h vs SE 7d	***
SE + DA3 12 h vs SE 7d	***	SE 12 h vs SE + DA3 7d	**
SE 1d vs SE 3d	***	SE + DA3 12 h vs SE 1d	**
SE 1d vs SE + DA3 3d	**	SE + DA3 12 h vs SE 3d	***
SE 1d vs SE 7d	***	SE + DA3 12 h vs SE + DA3 3d	***
SE 1d vs SE + DA3 7d	***	SE + DA3 12 h vs SE 7d	***
SE + DA3 1d vs SE 3d	***	SE + DA3 12 h vs SE + DA3 7d	***
SE + DA3 1d vs SE + DA3 3d	***	SE 1d vs SE 3d	***
SE + DA3 1d vs SE 7d	***	SE 1d vs SE + DA3 3d	***
SE 3d vs SE + DA3 3d	***	SE 1d vs SE 7d	***
SE 3d vs SE + DA3 7d	***	SE + DA3 1d vs SE 3d	***
SE + DA3 3d vs SE 7d	***	SE + DA3 1d vs SE + DA3 3d	***
SE 7d vs SE + DA3 7d	***	SE + DA3 1d vs SE 7d	***
		SE + DA3 1d vs SE + DA3 7d	**
		SE 3d vs SE + DA3 3d	*
		SE 3d vs SE 7d	***
		SE 3d vs SE + DA3 7d	***
		SE + DA3 3d vs SE 7d	***
		SE + DA3 3d vs SE + DA3 7d	***
		SE 7d vs SE + DA3 7d	***

3.5. Mitochondrial apoptosis markers Bax and Bcl-2

SE is neurotoxic and triggers apoptosis, which further contributes to increased epileptogenesis. We analyzed two key biomarkers for mitophagy and apoptosis. Compared to control animals, the levels of pro-

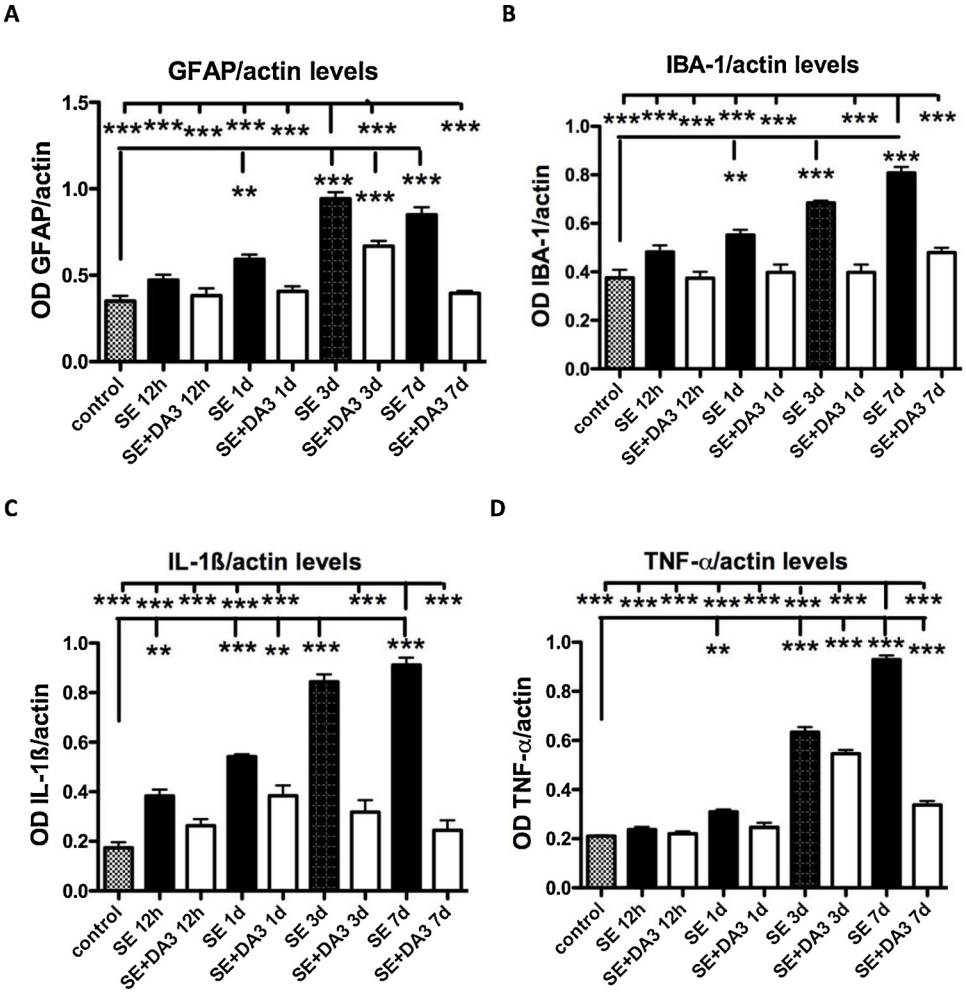


Fig. 3. The chronic inflammation response is reduced by DA3-CH, western blot analysis of protein levels. 3A: Levels of the biomarker GFAP for activated astrocytes. A one-way ANOVA found a difference between all groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE at day 3 vs. all other groups. For further results, please refer to the results section. 3B: Levels of the biomarker for microglia IBA-1. A one-way ANOVA found a difference between all groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE at day 7 vs. all other groups. For further results, please refer to the results section. 3C: Levels of the pro-inflammatory cytokine IL-1ß. A one-way ANOVA found a difference between all groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE at day 7 vs. all other groups. For further results, please refer to the results section. 3D: Levels of the pro-inflammatory cytokine TNF-α. A one-way ANOVA found a difference between all groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE at day 7 vs. all other groups. For further results, please refer to the results section. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. For sample western blots see Fig. 4C.

apoptotic mitochondrial Bax were much increased by 2.8-fold change at 3d after SE as analysed by western blot ($p < 0.001$). DA3-CH reduced levels significantly by 52% change($p < 0.001$). A one-way ANOVA found a difference between groups ($p < 0.0001$). Tukey post-hoc tests found significant differences between groups (see Fig. 4A).

Groups:	p-value:
control vs SE 1d	**
control vs SE 3d	***
control vs SE 7d	***
control vs SE + DA3 7d	*
SE 12 h vs SE 3d	***
SE 12 h vs SE 7d	***
SE + DA3 12 h vs SE 1d	*
SE + DA3 12 h vs SE 3d	***
SE + DA3 12 h vs SE 7d	***
SE + DA3 12 h vs SE + DA3 7d	*
SE 1d vs SE 3d	***
SE 1d vs SE 7d	*
SE + DA3 1d vs SE 3d	***
SE + DA3 1d vs SE 7d	***
SE 3d vs SE + DA3 3d	***
SE 3d vs SE 7d	*
SE 3d vs SE + DA3 7d	***
SE + DA3 3d vs SE 7d	***
SE 7d vs SE + DA3 7d	**

Compared to control animals, the levels of anti-apoptotic mitochondrial Bcl-2 were increased by a 2.6-fold change at 3d after SE as analysed by western blot ($p < 0.001$). DA3−CH increased levels significantly by a 1.6-fold change ($p < 0.001$). A one-way ANOVA found a difference between groups ($p < 0.0001$). Tukey post-hoc tests found significant differences between groups (see Fig. 4B).

Groups:	p-value:
control vs SE + DA3 1d	***
control vs SE 3d	***
control vs SE + DA3 3d	***
control vs SE 7d	***
control vs SE + DA3 7d	***
SE 12 h vs SE + DA3 1d	**
SE 12 h vs SE 3d	***
SE 12 h vs SE + DA3 3d	***
SE 12 h vs SE 7d	***
SE 12 h vs SE + DA3 7d	***
SE + DA3 12 h vs SE 3d	***
SE + DA3 12 h vs SE + DA3 3d	***
SE + DA3 12 h vs SE 7d	***
SE + DA3 12 h vs SE + DA3 7d	***
SE 1d vs SE 3d	***
SE 1d vs SE + DA3 3d	***
SE 1d vs SE 7d	***
SE 1d vs SE + DA3 7d	***
SE + DA3 1d vs SE 3d	***
SE + DA3 1d vs SE + DA3 3d	***
SE + DA3 1d vs SE 7d	**
SE + DA3 1d vs SE + DA3 7d	***
SE 3d vs SE + DA3 3d	***
SE 3d vs SE + DA3 7d	***
SE + DA3 3d vs SE 7d	***
SE + DA3 3d vs SE + DA3 7d	*
SE 7d vs SE + DA3 7d	***

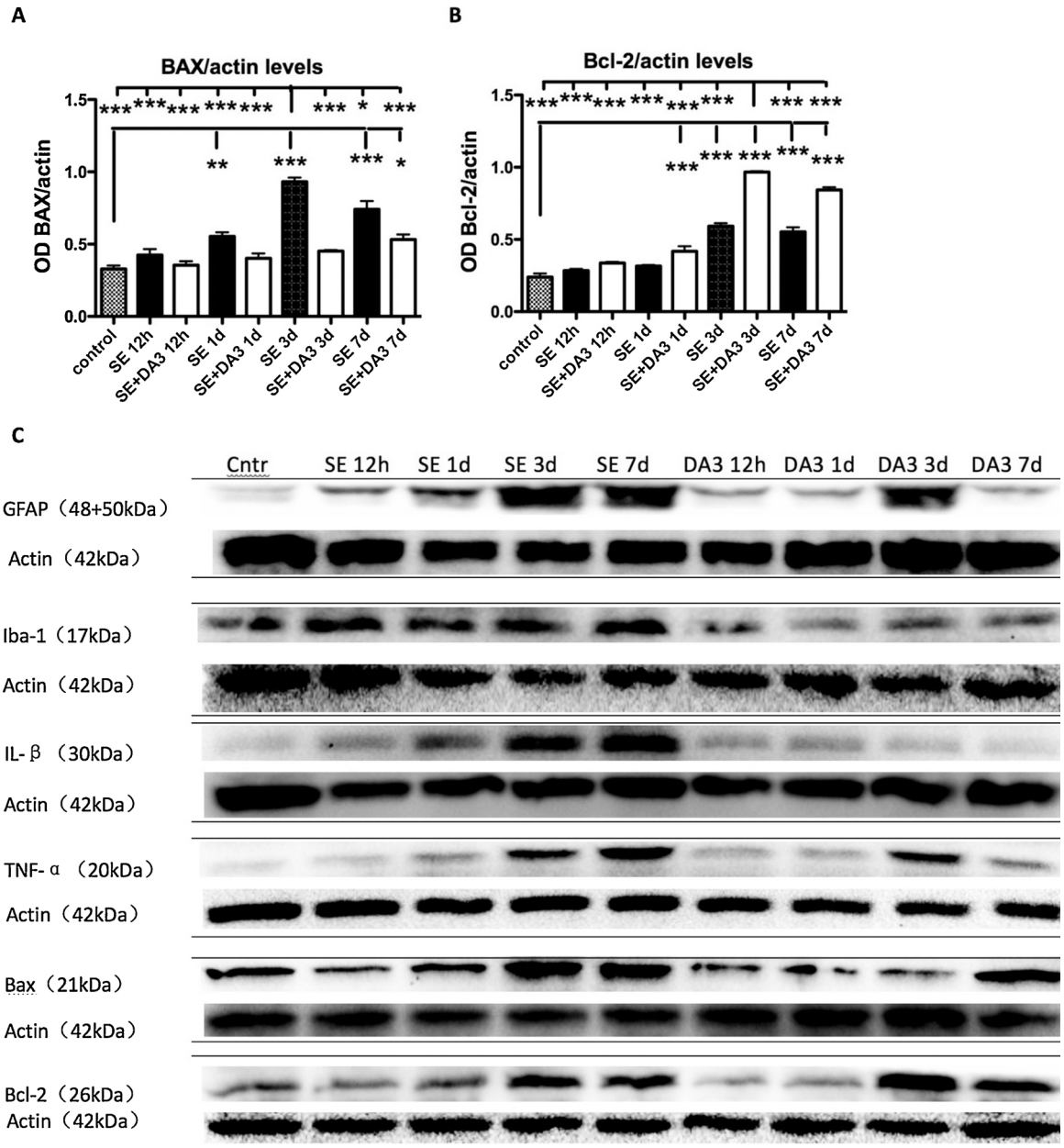


Fig. 4. Mitochondrial apoptosis markers BAX and Bcl-2. 4A: BAX levels. A one-way ANOVA found a difference between all groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE at day 3 vs. all other groups. For further results, please refer to the results section. 4B: Levels of Bcl-2. A one-way ANOVA found a difference between all groups. Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE + DA3-CH at day 3 vs. all other groups. For further results, please refer to the results section. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. 4C: sample western blots.

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.01$

3.6. Neuronal numbers in area CA1 of the hippocampus (NeuN positive cells)

Neuronal nuclei (NeuN), a specific marker for mature neurons, was used to assess neuronal loss. A one-way ANOVA found a difference between groups when analyzing the numbers of NeuN positive cells in area CA1 ($p < 0.0001$). Tudey multiple comparison post-hoc tests found that SE greatly reduced neuronal numbers at 7d by 66% change compared to control animals ($p < 0.001$). Treatment with DA3−CH protected neurons to some degree and neuronal numbers were increased by 2.7-fold change ($P < 0.001$)(Fig. 5).

Groups:	p-value:
control vs SE 12 h	***
control vs SE + DA3 12 h	***
control vs SE 1d	***
control vs SE + DA3 1d	***
control vs SE 3d	***
control vs SE + DA3 3d	*
control vs SE 7d	***
SE 12 h vs SE 3d	*
SE 12 h vs SE + DA3 3d	*
SE 12 h vs SE 7d	***
SE 12 h vs SE + DA3 7d	***
SE + DA3 12 h vs SE 3d	**
SE + DA3 12 h vs SE 7d	***
SE + DA3 12 h vs SE + DA3 7d	**
SE 1d vs SE + DA3 1d	**
SE 1d vs SE + DA3 3d	***

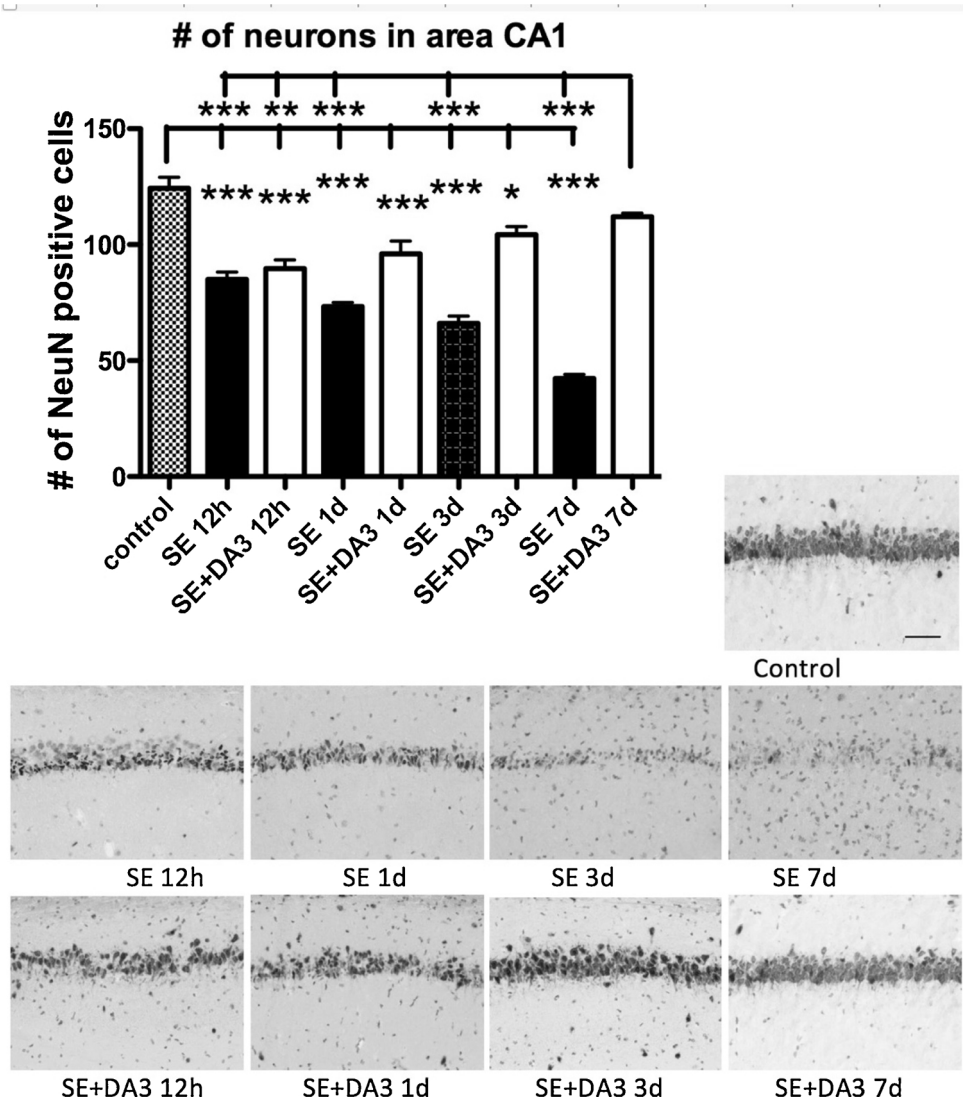


Fig. 5. Quantification of neurons in area CA1 of the hippocampus. A one-way ANOVA found a difference between all groups in numbers of NeuN -positive cells ($p < 0.0001$). Post-hoc Tukey multiple comparison tests showed differences between groups. Shown are differences between control vs. all other groups, and SE + DA3-CH at day 7 vs. all other groups. For further results, please refer to the results section. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Scale bar in control micrograph = 40 μm.

SE 1d vs SE 7d	***
SE 1d vs SE + DA3 7d	***
SE + DA3 1d vs SE 3d	***
SE + DA3 1d vs SE 7d	***
SE 3d vs SE + DA3 3d	***
SE 3d vs SE 7d	**
SE 3d vs SE + DA3 7d	***
SE + DA3 3d vs SE 7d	***
SE 7d vs SE + DA3 7d	***

4. Discussion

Our study demonstrates that the dual-GLP-1/GIP receptor agonists DA3 − CH has neuroprotective properties in the pilocarpine-induced epilepsy rat model. The results showed that treatment with DA3 − CH after initiation of SE suppressed chronic inflammation, the release of pro-inflammatory cytokines, reduced mitochondrial apoptosis/mitophagy, and neuronal loss in the CA1 area of the hippocampus compared to the SE saline treated group. In addition, in the analysis of the SE-induced pathology, it became clear that the drug treatment had ameliorated the development and pathological outcomes of SE. These effects underpin the anti-epileptogenic activity of dual-GLP-1/GIP receptor agonists DA3 − CH.

The most classic case of acquired and relapsing epilepsy is temporal lobe epilepsy (TLE), which originates from the hippocampal formation, a structure situated in the mesial temporal lobe (Majores et al., 2007). Our experiment used the prominent pilocarpine-induced TLE model. Systemic administration of pilocarpine, a cholinergic muscarinic agonist, is extensively used as an animal model of SE because it can reproduce many of the features of SE, including selective interneuron loss, refractory seizures and poor suppression of seizures by anticonvulsants. Experimental evidence has demonstrated that the function of pilocarpine is by activating the M1 muscarinic receptor subtype, which disrupts the balance between inhibitory and excitatory transmission, and which causes the generation of SE. Animal studies have shown that both recurrent seizures and SE can damage the brain, especially the hippocampal CA1 area (Mendez-Armenta et al., 2014). In our study, we observed the pathophysiological changes in the hippocampal CA1 area after SE. Glia cells significantly contribute to the pathophysiology of seizures (Devinsky et al., 2013; AFA et al., 2009). The damage of glia could cause the initiation, development and establishment of epileptogenesis (Wetherington et al., 2008). Abnormal glia, including activated astrocytes and microglia, are a conspicuous feature

of epileptic foci in experimental epilepsy models and in the human brain (Wang et al., 2015; Somera-Molina et al., 2009). In our experiment, we detected elevated levels of GFAP and Iba-1 at diverse timepoints after SE. The results showed that SE markedly activated astrocytes and microglia compared to the control group. Activated glia-mediated inflammatory changes, excessive release of proinflammatory molecules, including IL-1β and TNF-α, can facilitate epileptogenesis (Devinsky et al., 2013; Vezzani et al., 2008; Volterra and Meldolesi, 2005). More specifically, these proinflammatory cytokines can lower the seizure threshold and change neuronal excitability, thus supporting the formation of a chronic neuronal network hyperexcitability which will produce SRS (Vezzani et al., 2013, 2011). During the course of epileptogenesis, seizure and inflammatory reaction interact. The inflammatory response can induce or facilitate the occurrence of seizure, and the seizure in turn will activate the release of inflammatory cytokines which further activate glia. This is a vicious circle that can maintain epileptogenesis and enhances the process of epilepsy. In our experiment, we also observed increased expression of IL-1β and TNF-α especially at 7d after SE. Furthermore, the persistent excitation of neuronal cells during SE can generate profuse reactive oxygen species and reactive nitrogen oxygen inducing mitochondrial dysfunction in the hippocampus (Cardenas-Rodriguez et al., 2013; Shin et al., 2011; Chen et al., 2010). The mitochondrial Ca^{2+} and ROS/RNS generation combine to open the mitochondrial permeability transition pore, a channel across the mitochondrial inner and outer membranes. Then, proapoptotic molecules could move from the mitochondria to the cytoplasm after MPTP treatment, which initiate the apoptotic pathways. One family of mitochondrial-associated proteins are the Bcl-2 peptides that consists of both antiapoptotic (Bcl-2, Bcl-xl, and Bcl-w) and proapoptotic (Bad, Bax, and Bim) members. The apoptotic signaling pathway is modulated by both pro- and anti-apoptotic molecules controlling or interacting with the pilocaroine SE –induced effects on membranes (Henshall and Engel, 2013). In our study, we observed that SE reduced the expression of Bcl-2 and increased expression of Bax. This can lead to mitophagy and eventually to neuronal loss (FD et al., 2000). During epileptogenesis, Glial activation and the excessive release of pro-inflammatory cytokines, as well as oxidative stress and the abundant production of free radicals, may take account for cell death via either an apoptotic or a necrotic pathway, reducing the number of neurons (Block et al., 2007). Consistent with previous studies, we also found progressive neuronal loss after SE in our experiment (Wang et al., 2015).

Importantly, we observed that treatment with DA3-CH could reduce the pathophysiological processes in the hippocampal CA1 area after SE. At present, the unimolecular dual incretin receptor agonist has been developed as a treatment for type 2 diabetes, and exhibits enhanced insulinotropic and anti-hyperglycemic efficacy relative to mono-agonist in diabetic animals (Finan et al., 2013). As shown previously, the incretins do not affect blood glucose levels in non-diabetic animals or humans (Yuan et al., 2017; Faivre et al., 2012; George et al., 2014). In our experiment, we also demonstrated that DA3－CH has no effect on blood glucose levels of non-diabetic rats. Importantly, GLP-1/GIP dual agonists can cross the BBB and activate simultaneously the corresponding receptor in the CNS (Hölscher, 2018). Previous studies have indicated that the unimolecular dual incretin exert neuroprotective effect by inhibiting glial activation and neuroinflammation, suppressing oxidative stress and apoptosis, protecting the number and function of synapses, and regulating neurotransmitter release, more effectively than did selective GLP-1 mono-agonist in CNS diseases including stroke, PD and AD (Hölscher, 2018). In our study, we found that the DA3-CH -treated group showed lower levels of GFAP, Iba-1, IL-1β, TNF-α, demonstrating a much reduced chronic inflammation response, and lower Bax levels along with higher levels of Bcl-2 to demonstrate improved mitochondrial function. These means that treatment with DA3-CH significantly mitigated SE-induced glial activation, neuroinflammation, cell apoptosis and neuronal loss after SE. Importantly, neuronal cell loss in area CA1 of the hippocampus was much reduced by DA3-CH, underscoring the neuroprotective effect of DA3-CH.

In conclusion, our results demonstrated that the novel GLP-1/GIP dual agonist DA3-CH shows promise to exert antiepileptogenic effects by attenuating SE-induced acute glial cell activation, neuro-inflammation, cell apoptosis and neuronal loss in the rat pilocarpine-induced SE model. Further studies are necessary to objectively record seizures by electroencephalograph (EEG) recording, and to record the frequency, duration, and severity of SRS by video monitoring and correlate this with the hippocampal pathophysiological changes of the chronic phase after SE. Our current study results support the concept that novel dual GLP-1/GIP receptor agonists show promise as a new therapeutic strategy for epilepsy.

Conflict of interest

Dr. Holscher is a named inventor on a patent submitted by Lancaster University UK on the use of dual GLP-1/ GIP analogues in neurodegenerative disorders. The other authors do not declare a conflict of interest.

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