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ORIGINAL PAPER

Intranasal Delivery of Botulinum Neurotoxin A Protects against Hippocampal Neuron Death in the Lithium-Pilocarpine Rat Model

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Abstract

Botulinum neurotoxins (BoNTs) block the release of a series of neurotransmitters, which are pivotal for neuron action. Intrahippocampal administration of BoNTs inhibits glutamate release, protects neurons against cell death, and attenuates epileptic seizures. Compared with intrahippocampal administration, intranasal delivery is less invasive and more practical for chronic drug administration. To assess whether intranasal administration is feasible, we examined the role of botulinum neurotoxin A (BoNT/A) in hippocampal neuronal injury after status epilepticus (SE) induced by pilocarpine. Our data showed BoNT/A could bypass the blood–brain barrier (BBB) and entered the olfactory bulb and hippocampal neurons. In addition, SE could result in up-regulation of pro-apoptotic proteins (Caspase-3, Bax), down-regulation of anti-apoptotic protein Bcl-2 and neuronal death in hippocampus. BoNT/A could suppress the expression of Caspase-3 and Bax, attenuate the decrease of Bcl-2, and inhibit hippocampal neuron death induced by SE. Meanwhile, there was no significant difference in cognitive behavior between the BoNT/A-pretreated rats and normal rats. Thus, we provided a more convenient and less invasive route for taking advantage of BoNT/A in the field of anti-epilepsy.

Keywords Botulinum Neurotoxin A · Intranasal delivery · Epilepsy · Olfactory nerve pathway · Apoptosis

Introduction

Epilepsy is a group of neurologic disorders characterized by paroxysmal, excessive and hypersynchronous discharge of neurons in the brain, which causes seizures or periods of unusual behaviors, sensations, and sometimes impairs awareness [1, 2]. Previous pharmacological experiments have shown that epilepsy involves the blocking synaptic and voltage-gated inhibitory conductance [3] or the activating synaptic and voltage-gated excitatory conductance [4]. Although more than 20 antiepileptic drugs have been used, nearly 20–30% patients still suffer from seizures and develop into refractory epilepsy. Therefore, there is still a need to further explore drugs for the treatment of epilepsy.

The eight types of BoNTs, named BoNT/A-H, cleave the soluble N-ethylmaleimide–sensitive fusion protein attachment protein receptors (SNAREs). SNAREs are the basic proteins that mediate vesicle transport and can be divided into two categories: vesicle SNAREs (v-SNAREs) and target membrane SNAREs (t-SNAREs) [5]. The presynaptic neuron SNARE complex contains three SNAREs, including Syntaxin1, SNAP-25 and vesicle-associated membrane protein (VAMP), where VAMP acts as t-SNARE, while Syntaxin1 and SNAP-25 act as v-SNAREs. BoNT/A cleaves SNAP-25, and has the longest duration of action, lasting as long as 160 days [6, 7]. BoNTs inhibit a variety of neurotransmitters, including acetylcholine, glutamate, gamma-aminobutyric acid (GABA), noradrenaline, glycine, dopamine, and serotonin [8]. Compared with glutamatergic

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neurons, hippocampal GABAergic synapses lack SNAP-25 and are more resistant to the action of BoNT/A and BoNT/E [9, 10]. Thus, they have more inhibition on excitatory postsynaptic currents [11, 12]. Previous studies have shown that intrahippocampal administration of BoNT/E inhibits glutamate release, blocks spike activity of pyramidal neurons, protects neurons against cell death, and reduces epileptic seizures [13, 14]. Similarly, BoNT/A and BoNT/B also play an antiepileptic role via intrahippocampal administration [15, 16], which is invasive and not practical for chronic drug administration.

To our knowledge, intranasal administration can bypass the BBB, which provides a less invasive and convenient way to the central nervous system [17]. However, the effect of intranasal administration of BoNT/A on hippocampal neurons in an epileptic model has not been studied. The present study investigated whether intranasally administered BoNT/A can be transported to the hippocampus and play a protective role against neurons death induced by epileptic seizures.

Materials and Methods

Animal Model

Adult male Sprague–Dawley rats (6–7 weeks, weighing 220–230 g) provided by Experimental Animal Center of Zhengzhou University were placed at room temperature (20 ± 2 °C), with controlled illumination (12 h light and 12 h dark cycle) and free access to food and water. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Zhengzhou University and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

The rats were administered with lithium chloride intraperitoneally (i.p.) (3 mEq/kg, Sigma, USA). Twenty h later, they received pilocarpine (30 mg/kg, i.p., Sigma, USA), and the control group rats received the same volume of 0.9% saline. To limit pilocarpine peripheral effects, Scopolamine Hydrobromide (1 mg/kg) was injected subcutaneously 30 min before administration of pilocarpine. According to Racine [18], the sustained presence of stage 4–5 level was determined as SE. After 1 h of SE, 5% chloral hydrate (300 mg/kg, i.p.) was administered to terminate seizures. Rats were sacrificed 3 h, 8 h, 24 h, and 72 h after the onset of SE.

BoNT/A Administration and Experimental Groups

100 U of BoNT/A (Hengli, Lanzhou, China) was dissolved into 320 µl saline (0.9%). Referring to previous study [19], rats were anesthetized with 5% chloral hydrate (300 mg/kg, i.p.), then placed in supine position to keep their heads in the horizontal plane. 80 µl of BoNT/A (25 U) was distributed to one rat per day, and nose drops (8 µl) were given to one naris, alternating drops every 6 min between the left and right naris. During the administration, the mouth and opposite naris were blocked so the drops could be naturally inhaled. Intranasal administration continued for 7 days, with a 1 day break in the middle. Thus, a rat was given 150U of BoNT/A in total, while the saline group was given equal volume of saline (0.9%) intranasally. Meanwhile, the remaining rats were also anesthetized.

The rats were randomly divided into four groups: (1) control group (n = 24): normal rats, as described above; pilocarpine group (n = 52): lithium chloride-pilocarpine administered, as described above; saline group (n = 24): 0.9% saline intranasal administered, 45 days before pilocarpine; BoNT/A (n = 24): 150 U of BoNT/A intranasal administered, 45 days before pilocarpine.

Morris Water Maze

The rats were randomly chosen from the control group (n = 8) and the BoNT/A group (n = 8) on the 30th day after intranasal administration. Referring to previous study [13], a black circular pool (250 cm in diameter) was filled with water (22–26 °C), and a black circular platform (10 cm in diameter) was positioned 2.0 centimeters below the water surface. Before testing, rats were placed in the pool without the platform for 60 s to habituate to the environment. The rats were trained for 6 days. During the first 5 days (four trials per day), the rats were trained to escape onto the submerged platform from different starting positions. The time it took for the rats to find the platform was recorded, and the rats were allowed to stay on the platform for 10 s to get acquainted to the surrounding environment. If the rats did not find the platform within 60 s, they were guided onto the platform and allowed to stay on the platform for 10 s. On the 6th day, the platform was removed; the number of rats crossing the platform's location within 60 s was recorded. All experiments were conducted in a blinded manner.

Histopathology

As previous study [20], morphological changes of neurons were analyzed at 72 h after SE. The rats were intracardially perfused with 4% paraformaldehyde under deeply anesthesia. The brains were removed and embedded in paraffin. The paraffin-embedded brains were cut into sections (5 µm) coronally. Six sections were selected at intervals of 50 µm for Nissl staining with toluidine blue. Surviving hippocampal pyramidal cells were counted per 1 mm length in the

hippocampal CA3 region under a high magnification (400×) by a blinded investigator.

TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was chosen to detected the apoptotic neurons using an in situ apoptosis detection kit (Roche, Germany) at the same time point selected for Nissl staining. Briefly, the sections (5 μm) were permeabilized by proteinase K for 30 min. After blocked endogenous peroxidase, the sections were incubated in the TUNEL reaction mixture. After washed with PBS, the sections were visualized by using converter-POD, and then rinsed with DAB substrate. Finally, the sections were washed and stained with hematoxylin. The number of TUNEL-positive cells was blindly counted along the hippocampal CA3 region under a high magnification (400×).

Immunofluorescence

The rats randomly chosen from the control group and the BoNT/A group were deeply anesthetized by chloral hydrate (300 mg/kg, i,p.) and perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde. The rats' brains were carefully removed, post-fixed for 24 h in 4% paraformaldehyde at 4 °C, and sequentially dehydrated with 10%, 20%, and 30% sucrose solutions. Brain tissues were cut into coronal sections (40 μm) with a freezing microtome. Sections were blocked with 10% normal goat serum and then incubated overnight at room temperature with the anti-BoNT/A-cleaved SNAP-25 antibody (1:1000, GeneTex, USA), which specifically recognizes the BoNT/A cleaved SNAP-25 but not the whole protein [21]. The following day, sections were incubated with Cy3 AffiniPure-conjugated secondary antibody for 2 h at room temperature. Sections were washed in PBS and mounted with DAPI (Roche, Germany). Olfactory bulb and hippocampal neurons were observed using a microscope (Leica, Germany).

Western Blot

Referring to previous study [22], the proteins were separated by SDS–polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membranes. After blocking in 5% fat-free milk in Tris-buffered saline with Tween buffer, the membranes were incubated with primary antibodies Anti-Bcl-2 (1:1000, ab59348, Abcam), Anti-Bax (1:2000; ab182733, Abcam) and Anti-Caspase-3 (1:2000; ab90437, Abcam). The blots were incubated with HRP-conjugated secondary antibody (1:10,000, Zhongshan Biotech, China). Immunoreactivity was visualized by chemiluminescence kit and exposed to Amersham Imager 600 (GE, America). The relative densities of each protein band were quantified using ImageJ software.

Statistical Analysis

The data were presented as mean ± standard deviation (SD) and analyzed by SPSS 19.0 software. The Morris water maze data were statistically analyzed by repeated-measures two-way analysis of variance (ANOVA) and Student's t-test. The others were performed by one-way ANOVA following LSD-t. $p < 0.05$ was considered statistically significant.

Results

BoNT/A Could Reach Olfactory Bulb and Hippocampal Through Intranasal Administration

As BoNT/A specifically cleaved SNAP-25 protein into cleaved-SNAP-25 (cl-SNAP-25), generally, cl-SNAP-25 protein was identified as the maker of BoNT/A existence with bioactivity. In order to verify that BoNT/A could enter the central nervous system through intranasal administration, olfactory bulb and hippocampal tissues were selected for analysis. Immunofluorescence assay was used to detect cl-SNAP-25. The results showed that cl-SANP-25 was detected in the tissues of the BoNT/A group rats, while cl-SANP-25 was not found in the control group (Fig. 1), confirming the feasibility of intranasal administration.

Morris Water Maze

In order to determine whether BoNT/A can cause cognitive deficits, Morris water maze was used to analyze how much time the rats took to escape to the submerged platform as well the number of times the rats crossed the platform's location despite the platform's absence. In previous preliminary experiments, we confirmed that BoNT/A can reach the hippocampus on the 30th days after intranasal administration (data not shown). In the first five days, the escape latency to find the platform was evaluated. ANOVA analysis showed that all groups decreased the escape latency by a significant day effect (two-way ANOVA; $F = 309.54$, $p < 0.001$) and the absence of group × day interaction (two-way ANOVA; $F = 1.63$, $p > 0.05$) (Fig. 2a). There was no statistical difference in escape latency between the control and BoNT/A groups. On the 6th day, there was no statistical difference in the number of crossing the platform area between them (Student's t-test; $p > 0.05$) (Fig. 2b).

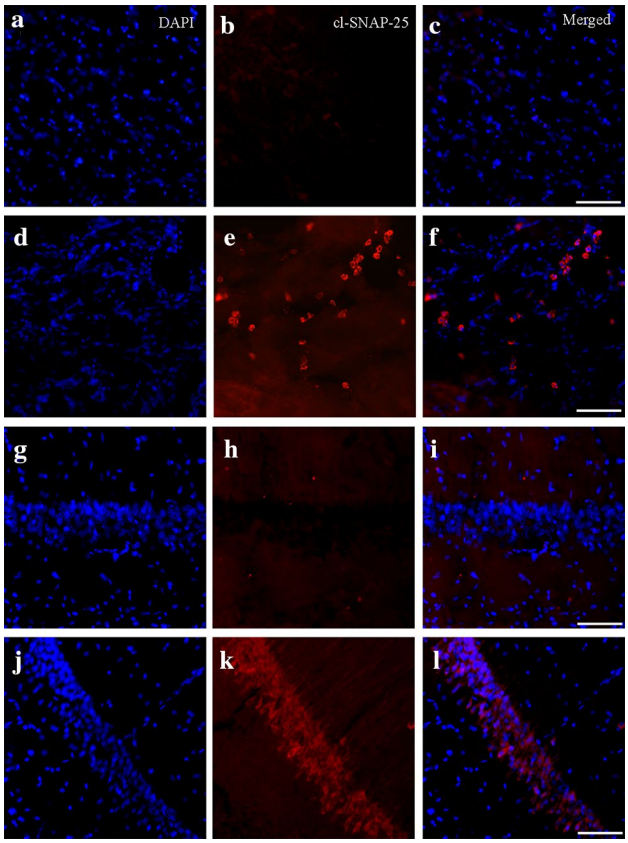

Fig. 1 Immunofluorescence of rats' olfactory bulb and hippocampus sections. **a, d, h** and **k** were DAPI stained, which illustrated the nuclei (blue); **b, e, i** and **l** were anti-BoNT/A-cleaved SNAP-25 stained (red); **c, f, j** and **m** were merged images, respectively. **a**–**f** are olfactory bulb tissues and **h**–**m** are hippocampus tissues; **a, b**, and **c** were in the control group, indicating that there was no cl-SNAP-25. **d, e**, and **f** were in the BoNT/A group, indicating the existence of cl-SNAP-25 in the neural cytoplasm. Furthermore, cl-SNAP-25 was also detected in the hippocampus tissue of the BoNT/A group (**k, l** and **m**) but not in the control group (**h, i** and **j**). Bar = 100 μm, n = 6/group. (Color figure online)

BoNT/A Protects Against Hippocampal Neuron Death Induced by SE

The hippocampal neurons were examined using Nissl staining for necrotic cell death and TUNEL staining for the apoptotic cell death induced by SE. Nissl staining showed that SE could cause severe neuron death 72 h after the termination of seizures. Compared with the control group, the number of surviving neurons was significantly decreased in the saline and the pilocarpine group ($p < 0.05$), while no significant difference was found in the number of surviving neurons between the saline and the pilocarpine group ($p > 0.05$). In addition, when compared with the saline groups, intranasal treatment of BoNT/A significantly prevented neuron death caused by SE ($p < 0.05$) (Fig. 3; Table 1). The result of TUNEL staining showed that the number of apoptotic cell was significantly increased in the saline and the pilocarpine groups in comparison with control group ($p < 0.05$), and no significant difference was found between them. On the other hand, in the rats pretreated with BoNT/A, the number of apoptotic cell was decreased compared with the pilocarpine group ($p < 0.05$) (Fig. 4; Table 2).

Effect of BoNT/A on the Expression of Bcl-2, Caspase-3 and Bax After Seizures

Our data shows that Bcl-2 protein levels started to decrease 3 h after SE ($p < 0.05$). Meanwhile, the protein levels of Caspase-3 and Bax began to increase statistically after 3 h ($p < 0.05$). Furthermore, the changes in the levels of the three proteins were more pronounced at the 24 hour than the 3 hour (Fig. 5a–c). Compared with the pilocarpine group, pretreatment of BoNT/A significantly suppressed the expression of Caspase-3 and Bax ($p < 0.05$), and significantly defended against the decrease of Bcl-2 in 24 h after SE ($p < 0.05$) (Fig. 5b–d). Meanwhile, there was no

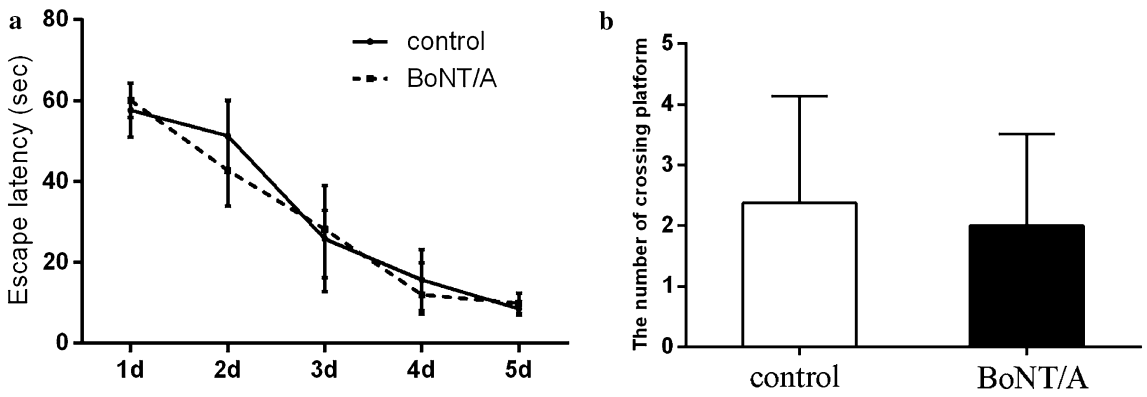

Fig. 2 Evaluation of spatial learning ability using Morris water maze. **a** the escape latency to the submerged platform in the first 5 days. No statistically significant difference was observed between the control and the BoNT/A group. **b** the numbers of rats crossing the platform area without platform on the 6th day. Meanwhile, no statistically significant difference was found between the two groups. n = 8/group

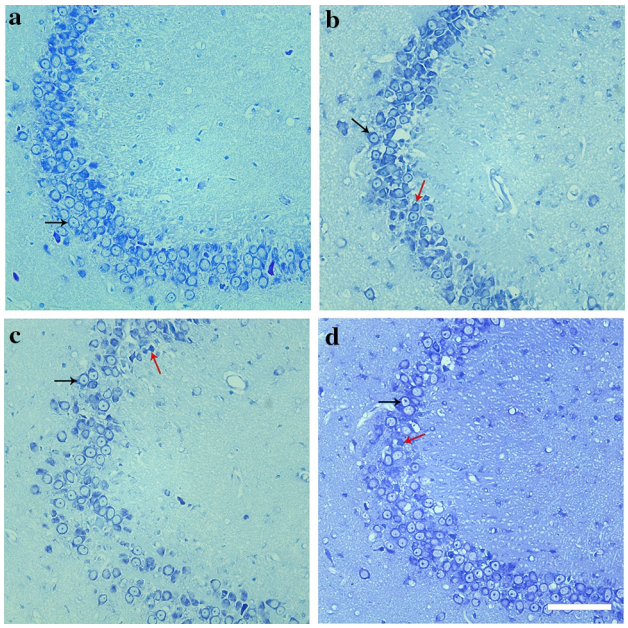

Fig. 3 The hippocampal CA3 region neurons stained with toluidine blue at 72 h after seizures (×200 magnification). **a** the control group, showing normal pyramidal neurons (black arrow). **b** the pilocarpine group, showing neuron loss and death (cytoplasm condensed and pyknotic nuclei, red arrow). **c** the saline group, showing neuron loss and death (cytoplasm condensed and pyknotic nuclei, red arrow). **d** the BoNT/A group, showing the protective effects of intranasal treatment of BoNT/A against neuron death caused by SE. Bar = 100 μm, n = 6/group. (Color figure online)

Table 1 Effect of BoNT/A on hippocampal neuron death induced by SE

Group	Number of surviving neurons (Mean ± SD)
Control	134.2 ± 10.1
Pilocarpine	69.5 ± 6.6*
Saline	60.1 ± 8.1*
BoNT/A	127 ± 9.3#

The number of surviving neurons per 1 mm length of the CA3 region of hippocampal were counted by light microscopy (400×). Data presented as Mean ± S.D

*$p < 0.05$ versus control group; #$p < 0.05$ versus the pilocarpine group

significant difference in the protein levels between the pilocarpine and the saline group.

Discussion

Current studies demonstrate that epileptic seizures trigger neuron loss and contribute to the presence of apoptotic signaling pathways [22, 23]. Additionally, apoptosis pathways were also found when analyzing the hippocampus and

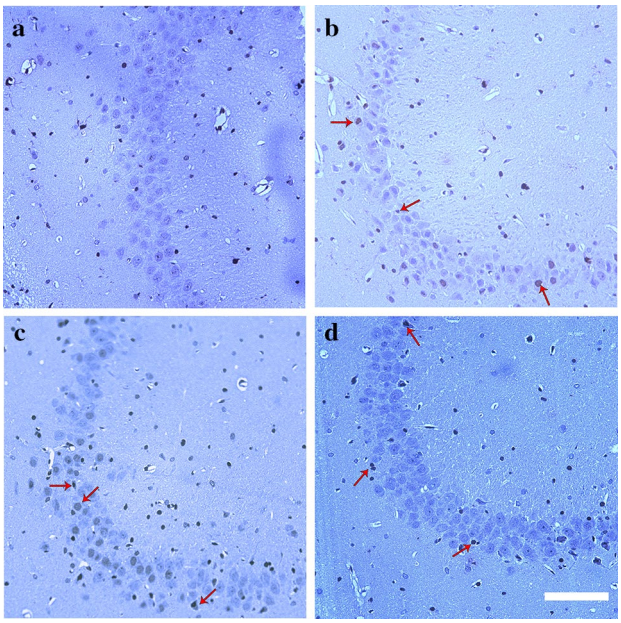

Fig. 4 TUNEL staining in hippocampal CA3 region neurons at 72 h after seizures (×200 magnification). **a** the control group. **b** the pilocarpine group, showing apoptotic cells (gray-brown cells, red arrow). **c** the saline group, showing apoptotic cells (gray-brown cells, red arrow). **d** the BoNT/A group, showing the decrease of the apoptotic neurons. Bar = 100 μm, n = 6/group. (Color figure online)

Table 2 Effect of BoNT/A on hippocampal neuron apoptosis induced by SE

Group	Number of apoptotic neurons (Mean ± SD)
Control	5.5 ± 1.0
Pilocarpine	22.2 ± 2.6*
Saline	24.5 ± 2.4*
BoNT/A	14.7 ± 1.6*#

The number of apoptotic neurons per 0.5 mm length of CA3 region of hippocampal were counted by light microscopy (400×). Data presented as Mean ± S.D

*$p < 0.05$ vs control group; #$p < 0.05$ vs the pilocarpine group

extra-hippocampal temporal cortex removed during surgery for intractable temporal lobe epilepsy [13]. In this study, it was found that BoNT/A can be delivered to the hippocampal neurons via intranasal administration and exerted protective effects against neuron death induced by SE.

The mechanisms underlying intranasal drug delivery to the CNS are complex, involving major components, such as the olfactory nerve, trigeminal nerve, cerebrospinal fluid, the vascular and lymphatic systems, among which olfactory nerve pathway is a major component [17]. Olfactory receptor neurons are projected to the primary olfactory cortex, piriform cortex, amygdala, and entorhinal cortex [24], and may allow biologicals to be targeted to specific regions of

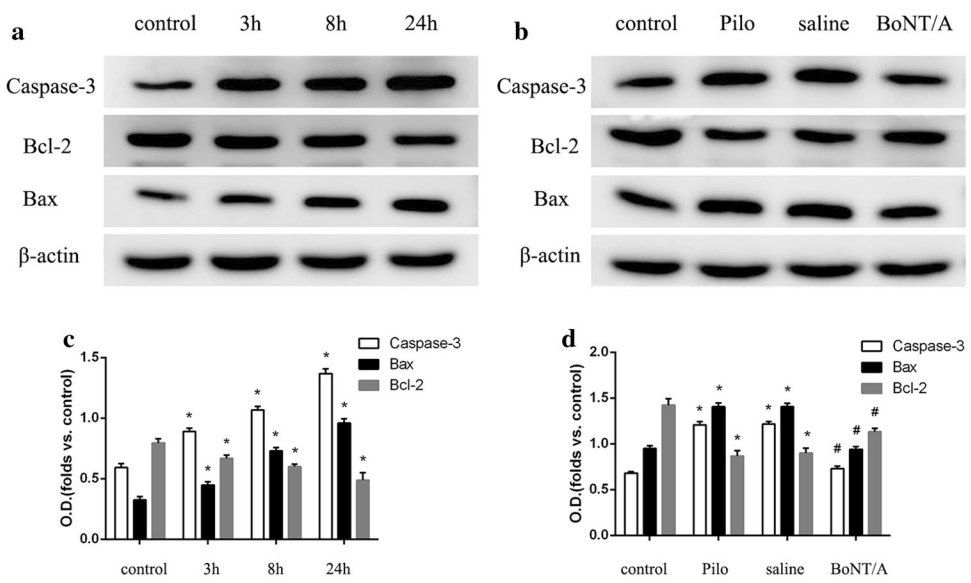

Fig. 5 The protein levels of Bcl-2, Caspase-3 and Bax. Western blot analysis (**a**) and quantitative analysis (**c**) of Bcl-2, Caspase-3 and Bax expression levels in hippocampus at a series of time points after SE. Western blot analysis (**b**) and quantitative analysis (**d**) of Bcl-2, Caspase-3 and Bax expression levels in hippocampus after intranasal administration. Date presented as Mean ± SD (N = 6/group). Pilo, pilocarpine. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus pilocarpine group

the brain [25]. MiR-146a, an important anti-inflammatory MicroRNA, delayed seizure onset in the lithium-pilocarpine mouse model via intranasal delivery [26]. BoNTs can be anterograde and retrograde transported via neuronal axons and have long distance effects [27]. After the injection of BoNT/A in the rats' unilateral hippocampus, cl-SNAP-25 was detected in the ipsilateral entorhinal cortex and ipsilateral hippocampus. In other parts of the cerebral cortex, such as the parietal area near the dorsal hippocampal injection site, cl-SNAP-25 was not detected. Analogous results were found in visual system, which indicates that BoNT/A was transcytosed to the next synapse and transported via neuronal axons with a limited actuating range [6]. Our data showed that cl-SNAP-25 was detected in olfactory bulb and hippocampal neurons after pretreatment of BoNT/A, indicating the feasibility of intranasal administration through the olfactory nerve pathway.

Caspases and Bcl-2 family proteins are the two major proteins regulating apoptosis. Caspase-3 is processed and activated by caspase 8, 9, and 10, and leads to apoptosis, both by extrinsic and intrinsic pathways [28, 29]. Comprised of more than 20 different members, Bcl-2 family proteins regulate cell death by inducing or inhibiting apoptosis [30, 31]. Bcl-2 promotes cellular survival by inhibiting the action of pro-apoptotic proteins, including Bak and Bax. Bax acts on the mitochondrial outer membrane to accelerate its permeabilization and release cytochrome C and reactivate oxygen species. The results show that the expression level of Bax and Caspase-3 increased in the first 24 h after epileptic seizures, coinciding with a decrease in Bcl-2, which concur with previous studies [23, 32]. Previous studies have shown intrahippocampal administration of BoNT/E inhibits glutamate release, blocks spike activity of pyramidal neurons, protects neurons against cell death, reduces acute seizures [13] and spontaneous seizures in chronic epileptic mice [14]. Meanwhile, Ilaria Manno found that BoNT/E inhibits hippocampal neuron injury as well as the activation of Caspase-3 induced by epileptic seizures, and speculated that the antiepileptic mechanism may involve apoptotic pathways [33]. Current research confirmed that BoNTs could inhibit the Ca^{2+}-dependent fraction of potassium-induced glutamate release, reduced glutamate excitotoxicity, and inhibited the activity of the membrane Na^{+} channel, which was responsible for the initial depolarization of action potentials in neurons [34]. Although our study suggests that BoNT/A's antiepileptic effects may interfere with apoptotic pathways, the comprehensive and specific content needs further study.

In conclusion, our study was the first to demonstrate that intranasal delivery of BoNT/A could protect against cell death induced by epileptic seizures, which may involve the suppression of apoptosis pathways. Therefore, our result suggest that intranasal administration of BoNT/A may be a more convenient and less invasive strategy for neuroprotection in epileptogenesis compared with intracranial injection.

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Compliance with Ethical Standards

Conflict of interest No conflicts of interest

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